UNIVERSITY
OF MICHIGAN
SEP 4 1958
MEDICAL
LIBRARY

JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

Volume 21
Number 2

August 1958

DEPOSITED BY THE
TED STATES OF AMERICA

Variation in Incidence of and Mortality from Stomach Cancer, with Particular Reference to the United States [1, 2]

WILLIAM HAENSZEL, *Biometry Branch, National Cancer Institute,*[3] *Bethesda, Maryland*

Summary

J. Nat. Cancer Inst. 21: 213–262, 1958.

Available evidence on the variation in stomach-cancer incidence and mortality with respect to time trends, age, sex, race, country, geographic latitude, nativity, socioeconomic class, and urban-rural residence is reviewed. In the search for exogenous factors that might influence the distribution of stomach-cancer cases, dietary effects appear to be the most obvious possibilities. The use of retrospective studies of cancer patients and matched controls to explore associations between stomach cancer and items of diet is discussed. Also included is a summary of variations in per capita consumption of several food items among population groups within the United States supplemented by observations for some countries with high stomach-cancer rates, which may be useful in providing leads for controlled, retrospective studies.

A growing number of observations on stomach cancer in man drawn from necropsy series, case registers, and death certificates has been accumulated and published. Much of this work was stimulated by and presented at meetings of the International Society of Geographical Pathology. The lack of a suitable experimental animal has undoubtedly enhanced interest in an epidemiologic approach to the study of this disease. Doll (*1*) has recently provided an excellent review of much of the epidemiologic data reported in the literature.

In some discussions there has been an implicit tendency to regard high or low rates for stomach cancer, or any disease, as a monolithic, national characteristic and not to probe further for patterns of variation within a country. More emphasis might be placed on variation in experience among population subgroups which may suggest further angles of research. For many years the American morbidity and mortality data

[1] Received for publication January 13, 1958.

[2] Part of this paper was presented at a symposium on gastrointestinal cancer held in Copenhagen during the week of July 14, 1958, under the auspices of the Committee on Geographical Pathology of the International Union against Cancer.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

on stomach cancer were quite deficient with respect to urban-rural, regional, and socioeconomic variation. Some of these deficiencies have now been remedied, and it seems profitable to review the current data for the United States to see how the findings fit with those reported elsewhere, and to advance some opinions on promising lines of investigation.

Materials

The original sources are cited when data on stomach cancer have been presented elsewhere. The results in text-figures 4 and 6 to 12 and tables 3, 4, 6, and 8 to 11 are published here for the first time. Tables 3, 4 (first half), 9, and 10 are based on special tabulations of the 1949–51 mortality data made by the National Office of Vital Statistics for the National Cancer Institute. Text-figures 6 to 12 and tables 4 (second half), 6, and 8, dealing with economic subregions and metropolitan and nonmetropolitan counties, are derived from tabulations of 1949–51 mortality data made for the Air Pollution Medical Program of the Public Health Service. I am indebted to Dr. Wilton M. Fisher for permission to use the latter material.

The United States data on consumption of foodstuffs, summarized in table 14, are from publications of the United States Department of Agriculture.

Since stomach-cancer incidence and mortality increase with age, age as a source of variation in these comparisons has been controlled either by use of age-specific rates or summary age-adjusted rates. Most of the age-adjusted rates, except those for economic subregions, were computed by the direct method, that is, the observed age-specific rates were weighted with reference to a standard population—the United States Census Population of 1950 was used for this purpose. The standardized mortality ratios for economic subregions were computed by an indirect method of age adjustment and represent the ratio of the known deaths to deaths expected for the area, assuming that the schedule of age-specific rates for the corresponding United States population prevailed.

Since mortality data afford the widest scope for comparisons, they are used as a principal axis for discussion despite some deficiencies in diagnostic accuracy and medical certification to which they are admittedly subject. The results have been checked by examination of cancer register and morbidity survey data where available. In general, the consistency among these various sources seemed very good; register and morbidity survey data and mortality data would independently lead to the same inferences. The deficiencies in cancer mortality data may have been overstressed and mortality data may prove reliable indicators for comparative purposes. In this connection two reports, which compared information on cancer site for the same individuals recorded in morbidity surveys and reported on death certificates, have found good correspondence between these two sources of information for each cancer site including the stomach (*2*, *3*).

International Comparisons

Extensive international comparisons of cancer mortality date from the work of Hoffman (*4*). More recently, Segi (*5*) has collected and published age-adjusted stomach-cancer mortality rates for 23 countries, and his results for males are rearranged and presented in table 1. The table also incorporates some results published by Doll (*1*), based on data collected by Neurdenberg (*6*). Doll used as his index for comparison an unweighted average of the age-specific death rates for males between the ages of 35 and 75. Segi's and Doll's materials yield almost the same ranking of countries. Differences in stomach-cancer death rates among countries remain fairly constant over a wide age range for each sex, and international comparisons for this site are little affected by the choice of a standard population for weighting the age-specific rates. Some measures of the relative frequency of stomach cancer among males, as reported by Dunham and Dorn (*7*), have also been included in table 1. While the relative

TABLE 1.—*Summary of international comparisons of mortality from stomach cancer*

Countries	Age-adjusted death rates per 100,000 males *† Segi (*5*)	Average death rate (*ages 35–74*) per 100,000 males‡§ Doll (*1*)	Relative frequency of stomach cancer among males‖ Dunham and Dorn (*7*)
Japan	67. 5	220	+++
Finland	65. 5	216	+++
Chile	65. 1	—	
Iceland	—	209	+++
German Federal Republic	45. 6	—	++
Norway	42. 1	131	+++
Switzerland	41. 4	—	+++
Italy	38. 0	—	++
Netherlands	37. 4	116	+++
Union of South Africa (whites)	36. 4	—	+
Belgium	33. 2	—	
Denmark	32. 5	96	+++
Sweden	31. 7	90	+++
Scotland	30. 8	—	
England and Wales	28. 3	91	++
Eire	28. 2	—	
Northern Ireland	28. 1	—	+++
France	27. 6	94	+++
Israel	27. 1	86	
Canada	24. 3	75	++
Australia	24. 1	—	++
United States (nonwhite)	23. 6	76	++
New Zealand	21. 4	—	
Venezuela	—	65	
United States (white)	16. 3	48	++

*Rates adjusted to the age distribution of the total population of 46 countries in 1950.

†Most of the mortality rates are for the years 1952–54; remaining rates are based on a combination of 1 or more years between 1951 and 1954, inclusive.

‡Average of age-specific rates for 5-year age groups.

§All mortality rates except for Iceland are based on a combination of 1 or more years between 1950 and 1953, inclusive; the Iceland rate is for the years 1940–49.

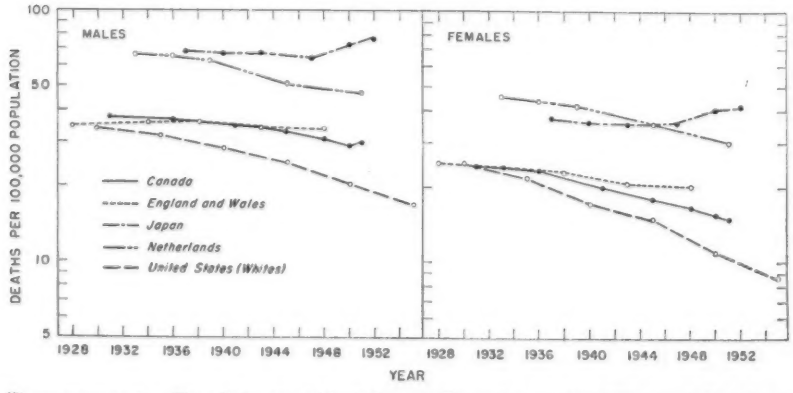
‖Percent of all male cancers: +++, 20 and over; ++, 7–19; +, 6 or less; results for some countries based on case register, survey, hospital, or necropsy statistics.

frequencies expressed as percentages of total cancers do not contradict the other results, they appear insensitive and do not discriminate among the West European countries to the same degree as mortality rates, which are related to a base population exposed to risk.

Anyone familiar with the operation of vital statistics registration systems and the difficulties in ensuring precise comparability among countries, in definitions, procedures, and medical certification practices, will disclaim an intention to read fine distinctions into the published mortality data. It must be conceded that the stomach-cancer certifications are subject to a greater margin of error than those for most other sites. However, they do have value as a yardstick for making broad, qualitative judgments, particularly when the pattern of stomach-cancer mortality rates agrees with evidence from other sources. Incidence data reported from cancer registers maintained in Denmark (*8*), Finland (*9*), New Zealand (*10*), and certain areas within the United States—California (*11*), Connecticut (*12*), and New York (*13*)—and from morbidity surveys in Japan (*14*) and the United States (*2, 15*) have been examined and found to be generally consistent with the mortality experience from these countries and to yield substantially the same rankings with respect to stomach-cancer risk.

The outstanding feature of table 1 is the low rate for whites in the United States. No other country with a well-established death registration system has a lower rate. This is a recent development attributable to the continued downward trend in stomach-cancer mortality in this country (text-fig. 1). As late as 1930 there was little difference between the rates for England and Wales and the United States.

The rates observed for Japan, Finland, Iceland, and Chile seem clearly higher than those for other countries. The high rates in Japan are supported by observations on mortality of Japanese in the United States and Hawaii reported by Smith (*16*) and Quisenberry (*17*). Steiner (*18*) also found a very high frequency of stomach cancer among Japanese in a



TEXT-FIGURE 1.—Trends in age-adjusted mortality rates per 100,000 population from stomach cancer by sex for 5 countries.

necropsy series from Los Angeles County General Hospital. Smith found the number of stomach-cancer deaths among Japanese men and women in this country to be 82 and 74 percent of the number expected on the basis of mortality experience in Japan. In the United States, the observed deaths for cancer of the intestines, including the rectum, were somewhat higher than those predicted on the basis of Japanese experience, particularly for males. However, while the mortality data from Japan may be inflated by overcertification of the stomach as a primary site, this factor could not of itself account for the high stomach-cancer mortality in Japan.

The high rate for Iceland and an intermediate rate for Norway coupled with the lower rates for Denmark and Sweden are suggestive. The Icelandic population comes from the same racial stock present in the other Scandinavian countries and the differences noted favor an environmental factor hypothesis.

The rates for the 4 separate registration areas within Great Britain and Ireland are in close agreement. The rates for Australia, Canada, and New Zealand, largely populated by immigrants from Great Britain, are all lower than in the mother country. A New Zealand report (*10*), which examines the separate experience for native-born and immigrants, is of interest. No difference was found in stomach-cancer mortality for the native-born and for the total immigrant population; the rate for persons migrating to New Zealand after age 30 was higher, though this particular result was not statistically significant. The Canadian provinces with sizable French-Canadian populations have higher stomach-cancer rates than those with predominantly English stock (*19*), so that the differential between Canada and England would be even greater if the comparison were restricted to the English-speaking provinces.

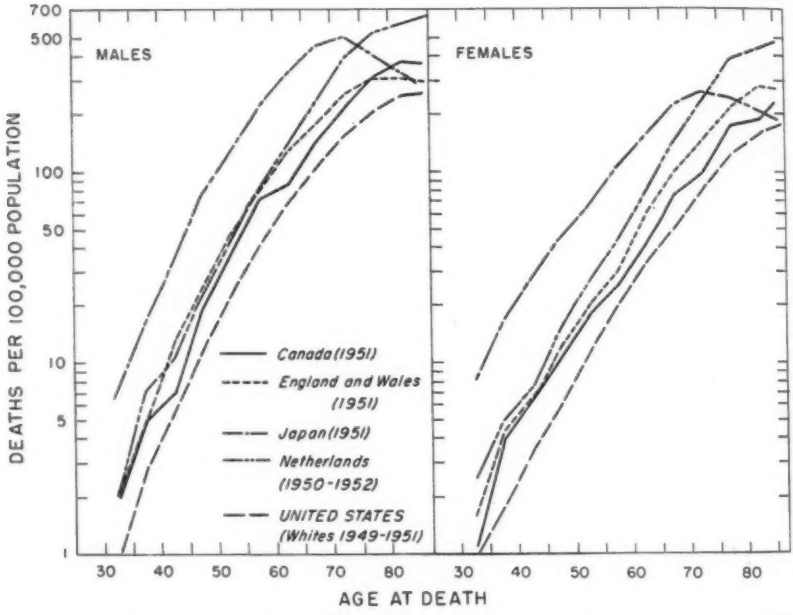
Only a small fraction of the Israeli population is native-born, and the rate for the total country conceals variation by country of origin. Jews migrating from North Europe seem to exhibit higher stomach-cancer rates than those coming from Asian countries (*20*).

Lack of knowledge concerning death registration practices and diagnostic facilities prevents detailed comment on the Venezuelan rate. Conceivably, the low stomach-cancer mortality may have some basis in fact, since there are hints of low risk in neighboring countries. Dungal (*21*) quotes Befarno as stating that stomach cancer was definitely rare in his autopsy material from Ecuador. In a series of cancer cases with histopathologic diagnoses from Antioquia (Colombia), Correa (*22*) reports a low relative frequency of the stomach as a primary site—slightly over 5 percent.

In general, countries in the higher latitudes appear to have higher rates, and Dungal (*21*) has commented on this fact. It would be interesting to inquire further as to whether this variation appears within countries as well, especially for countries like Chile and Norway covering a wide range in latitude. Data on this point for the United States are presented in the section dealing with regional variation.

Age and Sex

Age-specific death rates by sex for a recent time period are illustrated in text-figure 2 for the United States and 4 other countries with widely different stomach-cancer mortality experience (*6*, *23–25*). An arith-log scale was used so that parallel or straight lines denote equal rates of change with age. The relative differences among countries, as indicated earlier, remain fairly constant over a wide age span. The rates for each country fall into straight-line patterns from age 30 up to at least age 70; this property of a constant rate of increase with age is not uncommon and has appeared for some other cancer sites and other causes of death as well.



TEXT-FIGURE 2.—Age-specific mortality rates per 100,000 population from stomach cancer by sex for 5 countries.

The regular arrangement of age-specific rates when plotted on arith-log or log-log scales has been used by investigators (*26*) to develop or test models of carcinogenesis, a topic which will not be pursued here.

The apparent parallelism of the rates in text-figure 2 among countries with such divergent stomach-cancer experience is perhaps surprising. There is little difference between the male and female rates in this respect. Neurdenberg (*6*), who calculated values for the slopes of lines fitted to the age-specific rates plotted on a log-log scale for several countries and time periods, was impressed by the narrow range within which the results fell.

The stable arrangement of rates for each sex also implies a fairly constant sex ratio of rates. Doll (*1*) expressed the female mortality rates for

several countries as a percentage of the male rates and found most of them to be in the 50 to 65 percent range. We know of no country in which the female rate approaches or exceeds the male rate or even departs significantly from the range of values indicated by Doll. The national or state cancer registers and morbidity surveys cited earlier show sex ratios in conformity with those from the mortality data. In 1946, before extensive cancer register data were available, Barrett (*27*) had pointed out that some clinics treated a far higher proportion of male cases than indicated by the mortality data. In view of the later more broadly based register data, one may conclude that the figures for leading clinics probably reflected selective admission factors, the economic importance of the male breadwinner perhaps leading the family to a more zealous search for the best medical care. None of the 119 economic subregions of the United States (1949–51) show significant departures in sex ratios from the national data. The literature yields no unequivocally high female-male ratio for areas in other countries. The highest one noted was .85 for the Province of Limburg in the Netherlands—770 female deaths to 907 male deaths (*28*)—and even this result would require confirmation by observations for additional years to rule out the possibility of chance variation.

The drop in Japanese mortality at the oldest ages has been commented on by other writers. The prevailing opinion is that medical certifications of death may be less reliable for elderly Japanese. The Japanese rates might also be more seriously affected by competitive mortality from other causes, which assume greater importance after age 70. However, alternative hypotheses, including the possibility that the population of susceptibles may have been depleted by the high rates at earlier ages, cannot be dismissed completely.

Trends

Text-figure 1 shows the trend in age-adjusted death rates from stomach cancer during the past quarter century for 5 countries presenting widely different experience for this disease (*6*, *23–25*, *29*). As in text-figure 2 the rates have been plotted on an arith-log scale, so that equal slopes represent equal rates of change. The decline observed in the United States is not reproduced in all countries, though declines are not restricted to those with currently low rates. For example, the relatively high Netherlands' mortality has been falling at a rate about equal to that in the United States. Contrariwise, the mortality in England and Wales has changed little. The recent increase in the Japanese rate might be ascribed to changes in diagnostic and medical certification practices. For each country the rates of decline appear somewhat larger among females.

In the United States the drop in stomach-cancer mortality has accompanied a corresponding decrease for the total digestive system and the peritoneum (table 2). While other shifts have been recorded for sites within the digestive system—liver and biliary passages, and pancreas—the stomach has been the major factor in the downward trend noted for the total digestive system.

TABLE 2.—*Age-adjusted* mortality rates per 100,000 population from cancer of selected sites in the digestive system—United States, 1930–55*

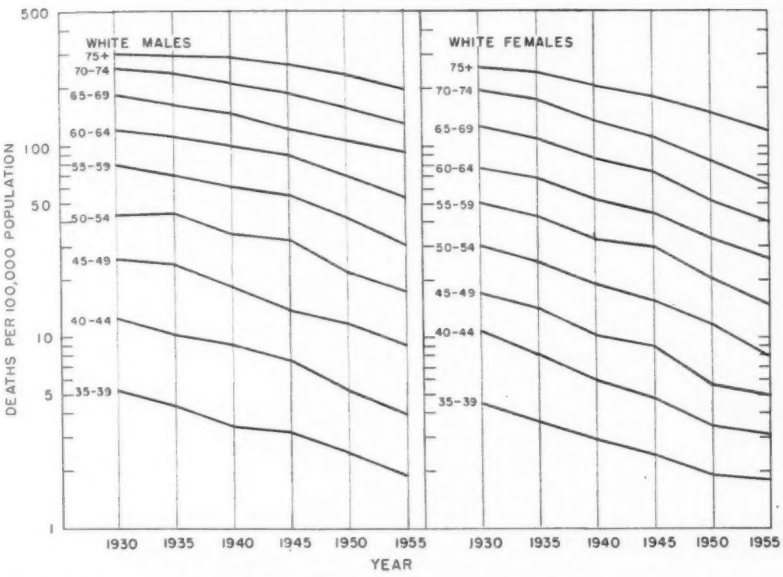
Site	Deaths per 100,000 population					
	1930	1935	1940	1945	1950	1955
Digestive system, total	65.0	64.5	62.2	61.7	54.0	50.3
Stomach†	28.8	26.5	22.5	19.9	16.0	13.0
Intestines†	12.3	14.0	15.5	16.9	15.2	15.1
Rectum‡	5.7	6.8	7.5	7.3	6.9	6.5
Esophagus	2.1	2.2	2.4	2.4	2.6	2.6
Pancreas	3.2	3.9	4.5	5.0	5.9	6.7
Liver and biliary passages	11.8	10.2	8.3	8.6	6.4	5.6

*Rates adjusted to age distribution in the United States 1950 census population.
†Duodenum included with stomach for 1930 and 1935 and with intestines for 1940 to 1955.
‡Anus included for 1930 to 1945, excluded for 1950 and 1955.

Although there are no comprehensive data on trends for regions or individual states, some differences in rates of change within the United States, particularly between North and South, may exist. In the 10-city surveys, stomach-cancer incidence in 4 northern cities declined 28 percent between 1937 and 1947, compared to 8 percent for 4 southern cities and 4 percent for 2 western cities (*see* table 11). On the other hand, the average annual rates of decline since 1930 in age-adjusted mortality have been almost identical in California and up-State New York (*11*, *30*). The Canadian mortality data indicate some differences among provinces with respect to time trends (*19*).

The available evidence from cancer register and morbidity survey sources supports the United States mortality picture of a reduced stomach-cancer rate. The age-adjusted incidence rates in Connecticut dropped 21 and 32 percent among males and females, respectively, between 1935 and 1940 and between 1947 and 1951 (*12*). The corresponding decline in up-State New York was 32 and 36 percent for the period 1942 to 1955 (*30*). The decline in age-adjusted incidence rates between the 10-city morbidity surveys of 1937 and 1947 was 20 and 28 percent (*31*). Most of these changes took place in the presence of stable incidence rates for total digestive system. The more recent incidence rates for each site have probably been raised by improved case reporting, and the comparison of register and survey data tends to underestimate the magnitude of decreases and may even convert small decreases into apparent increases. When the distribution of reported cases by primary site is examined by these sources, a decrease in the relative frequency of stomach cancer is noted. Breslow (*11*) has reported a decline in the relative frequency of the stomach as the primary site among cases reported to the California tumor registry in 1942–44 and 1951–52.

The decline in stomach-cancer mortality has been evident for each sex and age group in the United States (text-fig. 3). Among females the rate of decrease has been quite uniform for ages under 75; among males the shift has been more pronounced at ages under 65. The results offer a

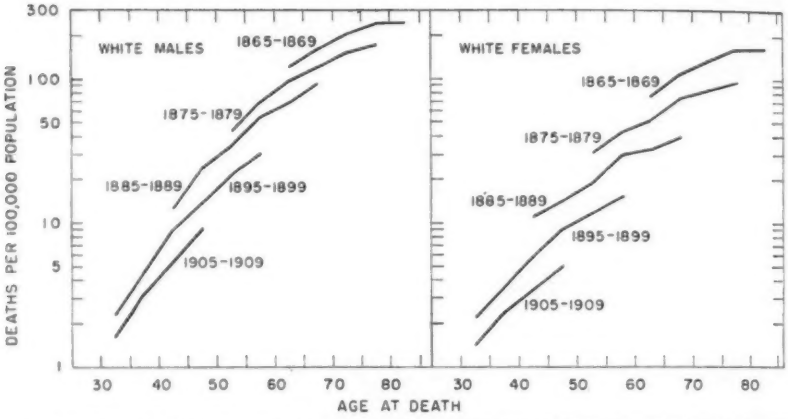


TEXT-FIGURE 3.—Age-specific mortality rates per 100,000 population from stomach cancer by sex. United States, whites, 1930–55.

striking departure from the experience of England and Wales, which has exhibited almost stable rates for males in each age group and only a modest decline for females (*29*).

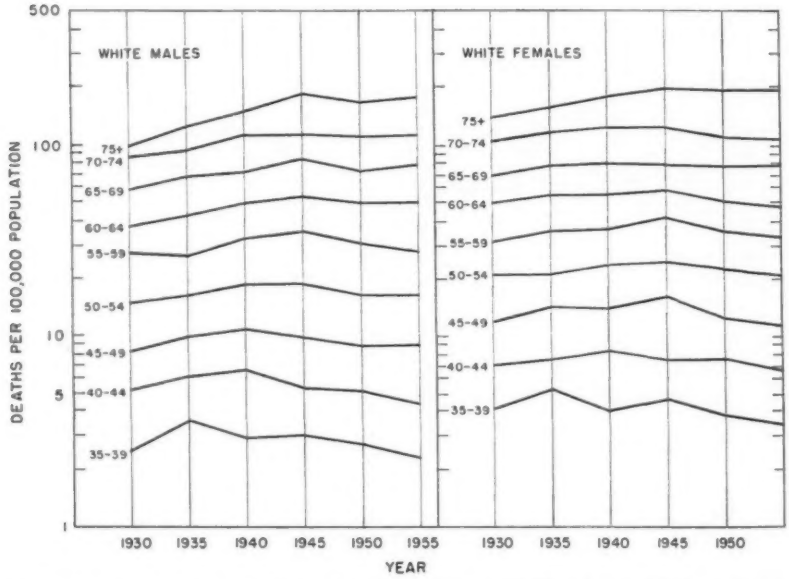
The rates from the cross sections of time are rearranged and plotted in text-figure 4 to represent the continuing experience for groups of persons born at approximately the same time (cohorts). The results, which could have been anticipated from text-figure 3, show a downward displacement in stomach-cancer death rates for successively younger cohorts of males and females in this country, with no indication that the changes will cease in the near future. As the younger cohorts grow older, further declines in mortality for the older age groups may be forecast. Again, the contrast with England may be emphasized. Stocks (*29*) and Case (*32*) examined the cohort structure of mortality from stomach cancer in England and found virtually no shift among males and only a slight displacement among females.

An interpretation of trends for the United States must consider whether the decline in risk is real or an artifact induced by changes in diagnostic procedures and concepts. If the earlier high rates for stomach cancer were grossly inflated by inaccurate certifications, some of the deaths should now be assigned to other rubrics. The more likely categories to be affected by such "transfers" are cancers of other sites within the digestive system, particularly of the intestines and pancreas. The trend of the age-specific rates provides evidence on this point. Text-figure 5 presents the data for the intestines. When examined by age group the trends for the



TEXT-FIGURE 4.—Death rates from stomach cancer per 100,000 population between 1930 and 1955 for cohorts born in successive decades. United States, whites.

stomach, intestine, pancreas, and liver and biliary passages (not shown) are not compensatory. For the younger ages it is particularly difficult to single out plausible possibilities for transfers from cancer of the stomach to other diseases within or without the digestive system. Another reason may be advanced for discounting the magnitude of such "transfer" effects. When the data for divisions (table 9) and economic subregions



TEXT-FIGURE 5.—Age-specific mortality rates per 100,000 population from cancer of the intestines by sex. United States, whites, 1930–55.

are examined, no compensatory relationship between the standardized mortality ratios for the stomach, intestines, and other sites of the digestive system appears.

Race

Negroes comprise more than 95 percent of the nonwhite population in the United States, and comparisons of whites and nonwhites drawn from vital statistics and morbidity survey sources reflect, principally, differences between whites and Negroes. Table 3 presents white and nonwhite age-adjusted cancer mortality rates, 1949–51, for selected primary sites. The stomach is one of the major sites where the cancer risk is higher among Negroes. It may be remarked that mortality for the upper portion of the alimentary canal, esophagus, and stomach is higher among Negroes than whites, while the relative standing is reversed for the intestine and rectum. The mortality data on race differences by site agree substantially with the findings from the 10-city morbidity surveys (*15*).

The Negro excess for stomach cancer persists in all regions of the country and in metropolitan and nonmetropolitan counties as well. The nonwhite figures for the Mountain and Pacific divisions are not relevant

TABLE 3.—*Age-adjusted* mortality rates per 100,000 population from cancer of selected sites by sex and color, with nonwhite-white ratios—United States, 1949–51*

Sites	Males			Females		
	White	Non-white	NW/W	White	Non-white	NW/W
Cervix uteri (171)†	—	—	—	9.6	22.1	2.30
Uterus, other and unspecified (172–174)	—	—	—	9.1	18.8	2.07
Esophagus (150)	3.9	6.9	1.77	1.0	1.6	1.60
Stomach (151)	20.5	27.3	1.33	11.1	14.2	1.28
Prostate (177)	15.7	19.2	1.22	—	—	—
Biliary passages and liver (155, 156.1)	5.9	7.0	1.19	7.0	5.6	.80
Pancreas (157)	7.3	6.1	.84	4.8	3.9	.81
Breast (170)	—	—	—	24.5	20.0	.82
Buccal cavity and pharnyx (140–148)	5.6	4.4	.79	1.3	1.7	1.31
Larynx (161)	2.3	1.8	.78	0.2	0.3	1.50
Lung and bronchus (162, 163)	20.5	15.7	.77	4.4	3.7	.84
Lymphosarcoma, *etc.* (200–203, 205)	6.6	4.8	.73	4.2	2.9	.69
Rectum (154)	8.2	5.9	.72	5.7	4.9	.86
Bladder and other urinary organs (181)	6.1	4.2	.69	2.6	2.7	1.04
Ovary (175)	—	—	—	7.6	5.0	.66
Intestines (152, 153)	14.9	9.2	.62	16.5	11.2	.68
Kidney (180)	3.1	1.8	.58	1.8	1.3	.72
Leukemia (204)	7.2	4.1	.57	5.0	2.8	.56
Skin (190, 191)	2.9	1.0	.34	1.8	1.0	.56

*Rates adjusted to the age distribution of the United States 1950 census population.
†International List Numbers, sixth revision, in parentheses.

to this point; Indians are the predominant nonwhite group in the Mountain States, while Chinese and Japanese form one third of the nonwhite population in the Pacific States. The regional contrast for nonwhites in table 4 may have been diluted by the recent, extensive, northward migration of Negroes from the South. No incidence or mortality data have ever been collected or presented separately for Negroes born in northern states.

TABLE 4.—*Age-adjusted* mortality rates per 100,000 population from stomach cancer by sex, color, division, and type of county, with nonwhite-white ratios— United States, 1949–51*

	Males			Females		
	White	Non-white	NW/W	White	Non-white	NW/W
United States	20.5	27.3	1.33	11.1	14.2	1.28
Division:						
New England	22.6	33.0	1.46	12.2	11.3	.93
Middle Atlantic	23.7	36.2	1.53	13.2	17.9	1.36
South Atlantic	15.6	26.6	1.71	8.5	13.7	1.61
East South Central	15.3	22.3	1.46	9.5	12.9	1.36
West South Central	16.5	26.2	1.59	9.2	14.0	1.52
East North Central	21.6	30.8	1.43	11.5	15.4	1.34
West North Central	20.5	28.5	1.39	11.0	14.6	1.33
Mountain	21.0	24.5†	1.17†	11.0	15.0†	1.36†
Pacific	20.0	32.4‡	1.62‡	9.7	13.4‡	1.38‡
Type of county:						
Metropolitan, with central city	22.2	31.3	1.41	11.7	14.6	1.25
Metropolitan, without central city	20.9	31.3	1.50	11.1	16.6	1.50
Nonmetropolitan	18.6	23.7	1.27	10.3	13.6	1.32

*Rates adjusted to age distribution of the United States 1950 census population.
†Indians are the predominant nonwhite group.
‡Chinese and Japanese constitute one third of the nonwhite population.

Smith (*16*, *33–35*) has published several papers on cancer mortality among minor racial groups within the United States and Hawaii. His findings on stomach cancer are shown in table 5. Cancer of this site is not uniformly higher among all Orientals, the mortality being considerably greater among Japanese and Hawaiians than among Chinese and Filipinos. Quisenberry (*17*) has compiled age-adjusted stomach-cancer mortality rates in Hawaii for the years 1941–50. His results coincide with those of Smith on the high Japanese and Hawaiian rates and the low Filipino rate, but show a slightly lower rate for Chinese than for whites.

The prevailing level of stomach-cancer mortality among Japanese in the United States, including Hawaii, is roughly four fifths of that currently observed in Japan, which supports the belief that the rates in Japan reflect a true increase in risk, but leaves unanswered the question whether they are due to innate biological characteristics of the Japanese or to the operation of environmental factors. Smith has also pointed out that the Japanese have significantly higher rates for stomach cancer in

TABLE 5.—*Standardized stomach-cancer mortality ratios (SMR)* for racial groups in the United States and Hawaii as reported by Smith (16, 33–35)*

Racial groups	Males	Females
Whites (1950–51)	100	100
Chinese (1949–52)	130	125
Japanese (1949–52)	308	317
Indians (1949–52)	98	72
Hawaiians (1949–52)	236	452
Filipinos (1949–52)	58	–

*The standardized mortality ratios for racial groups are expressed as percentages of the corresponding United States white rate after adjustment by the indirect method for age has been made.

Hawaii than in the continental United States, a relationship that does not exist for the Chinese. The phenomenon of progressively lower Japanese rates for stomach cancer from Japan to Hawaii to the United States also prevails for vascular lesions of the central nervous system and, in reverse, for diseases of the heart (*36*). More information on the extent to which stomach-cancer mortality may be modified by changing environment will become available as generations of Japanese born in the United States reach the ages where cancer is an important cause of death.

The results for the American Indians may be the least trustworthy and should be interpreted cautiously. The quality of medical certification and even the completeness of death registration have been poor among some of the larger Indian tribes and there is uncertainty about the correct population base for computing rates. Smith's results indicate marked deficits among Indians for most sites, including the total digestive system, compared with the experience of whites and nonwhites in the United States. The cervix and the liver and biliary passages are the only sites for which Indians clearly have higher rates. The rate for stomach cancer is close enough to that for whites so that a true excess or a negligible difference in risk for Indians might be converted into an apparent deficit if the Indian data were seriously affected by understatement of cancer deaths. Warwick and Phillips (*37*) have reported on cancer incidence among the Canadian Indians for the period 1948–52; they feel that the population and diagnostic data are reasonably adequate, though they express no opinion on how fully the government medical facilities are utilized by the Indians. They agree with Smith in showing that Indians have lower over-all cancer rates than Canadian whites, the cervix again being the outstanding exception. While they present no separate results for the stomach, their deficit for the total digestive system appears fully as large as that noted by Smith and would support the possibility of a stomach-cancer deficit among North American Indians.

Urban-Rural Residence

The best American information on urban-rural differentials in cancer incidence and mortality is contained in hitherto unpublished mortality

data for 1949–51 and in publications from the Connecticut and the New York State cancer registers (*12*, *13*) and the Iowa cancer morbidity survey (*2*).

Because of the heavy postwar migration to unincorporated suburban areas surrounding large cities, the 1949–51 mortality data did not use the classification of incorporated places over a specified size to form an urban-rural dichotomy, but relied instead on a coarser geographic grouping, a comparison of metropolitan and nonmetropolitan counties, with the further subdivision of metropolitan counties into those with and without central cities of 50,000 population or over. Table 6 presents standardized mortality ratios (SMR) from this source for the stomach and other sites within the digestive system. The SMR's for metropolitan counties with and without central cities do not differ greatly, the latter usually being slightly smaller. An urban-rural differential for stomach cancer, as measured by this particular contrast, is present but not as marked as for other sites in the digestive system. The stomach conforms to the experience for other primary sites in exhibiting a smaller metropolitan-nonmetropolitan gradient for females as compared to males.

These findings on differences between town and country in cancer risk are not confined to death certificates and cannot be attributed solely to varying practices of medical certification. Despite some differences in definitions of urban and rural areas, incidence data from cancer registers and morbidity surveys in 3 states, summarized in table 7, on the whole agree with the mortality figures, both on the small size of the urban-rural differential for stomach cancer and on the ranking of this site *vis-à-vis* other sites. Parenthetically, it may be remarked that the New York State data show similar urban-rural ratios for stomach cancer in both metropolitan and nonmetropolitan counties. The difference between Connecticut and Iowa in urban-rural ratios for stomach cancer is interesting, and raises the question whether the nature of the urban-rural relationship for stomach cancer varies with the section of the country.

Prior to the present compilation of the 1949–51 mortality data this point could not be investigated systematically because the annual vital statistics publications did not carry sufficient age detail on deaths (for individual primary sites) in states and smaller political subdivisions. Table 8 consolidates the 1949–51 stomach-cancer mortality data for the 119 economic subregions into 8 areas. This grouping of economic subregions, which cross state lines, was intended to approximate the familar Bureau of the Census arrangement of states into geographical divisions. The results indicate that the Connecticut and Iowa findings are not isolated special situations but conform to the more general results for the Northeast and West North Central areas. There are 4 areas in which the nonmetropolitan SMR's exceed those for metropolitan counties. The metropolitan-nonmetropolitan ratios are largest for those regions with heavy concentrations of foreign-born population. The economic subregions exhibiting the largest metropolitan-nonmetropolitan contrasts include those whose central cities are New York, Cleveland, Chicago, and

Vol. 21, No. 2, August 1958
469463—58——2

TABLE 6.—*Standardized mortality ratios (SMR)* from cancer of selected sites in the digestive system for metropolitan and nonmetropolitan counties, by sex—United States, whites, 1949–51*

Sites	White males				White females			
	Metropolitan counties		Non-metropolitan county	Metropolitan county with central city / Non-metropolitan county	Metropolitan counties		Non-metropolitan county	Metropolitan county with central city / Non-metropolitan county
	With central city	Without central city			With central city	Without central city		
Esophagus (150)†.....	211	166	87	2. 43	47	37	33	1. 42
Stomach (151).....	139	130	116	1. 20	73	69	65	1. 12
Intestines (152, 153)....	112	110	79	1. 42	115	122	96	1. 20
Rectum (154).....	151	141	84	1. 80	100	94	64	1. 56

*Standardized mortality ratios are expressed as percentages of the total United States rate (both sexes, all races) for each site, after adjustment by the indirect method for age.
†International List Numbers, sixth revision, in parentheses.

TABLE 7.—*Age-adjusted* cancer incidence rates per 100,000 population, from cancer of selected sites in the digestive system, by sex for urban and rural areas, as reported by Levin* et al. (13)

Primary site	Males			Females		
	New York† 1949–51	Connecticut† 1947–51	Iowa† 1950	New York 1949–51	Connecticut 1947–51	Iowa 1950
	Urban/rural ratio of rates					
Esophagus	1.78	2.35	1.86	0.92	2.14	0.50
Stomach	1.14	1.34	1.00	1.16	1.29	0.93
Intestine	1.34	1.46	1.45	1.15	1.28	1.23
Rectum	1.33	1.30	1.31	1.20	1.03	0.91
	Urban					
Esophagus	6.6	8.7	3.9	1.1	1.5	0.6
Stomach	23.7	28.5	24.7	11.7	15.5	13.1
Intestine	23.9	25.6	26.9	25.7	28.4	29.3
Rectum	18.3	20.1	16.3	11.9	14.0	10.0
	Rural					
Esophagus	3.7	3.7	2.1	1.2	0.7	1.2
Stomach	20.8	21.3	24.7	10.1	12.0	14.1
Intestine	17.9	17.5	18.6	22.3	22.2	23.8
Rectum	13.8	15.5	12.4	9.9	13.6	11.0

*Rates adjusted to age distribution of the United States 1950 census population.

†For New York State and Iowa, "urban" includes all incorporated places of 2,500 population and over, and certain other political subdivisions so classified by the U. S. Bureau of the Census under special rules relating to population size and density; for Connecticut, "urban" comprises 76 towns with high population density.

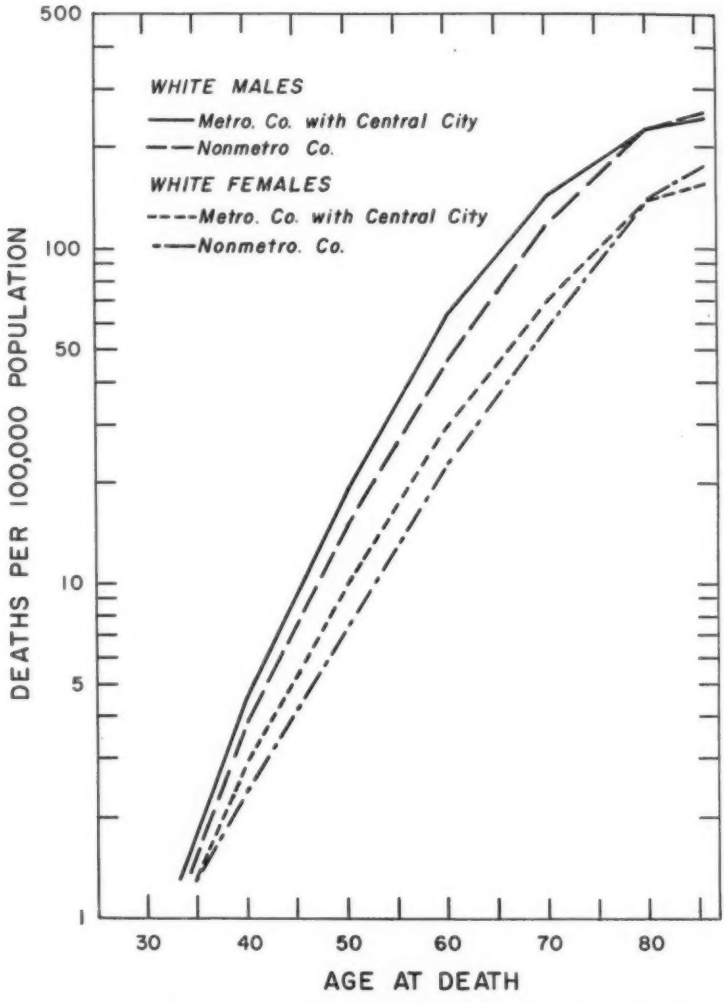
Los Angeles, all cities with sizable foreign-born groups. Since the foreign-born population is predominantly in the metropolitan counties, it seems quite likely that these ratios may be influenced by ethnic group differences. Lombard and Doering (*38*), in an earlier analysis of mortality in Boston over a 10-year period, found the foreign-born from certain countries to have much higher stomach-cancer rates than the native-born. The relatively small urban-rural differences might well be further reduced or even disappear if comparisons were restricted to the native-born. This point can be studied further when special tabulations of the 1950 cancer deaths by country of birth of the decedent, now under way, are completed.

Text-figure 6 contrasts the age-specific mortality rates (1949–51) for white males and females in metropolitan (with central city) and non-metropolitan counties. The urban excess is confined to ages under 75 and disappears or is reversed at older ages. Similar tendencies appear in the New York and Connecticut incidence rates and the stomach-cancer death rates for England and Wales (*39*). These tendencies bear a resemblance to that of the rates for the oldest age groups in some of the international comparisons. Whether the results imply the selective operation

TABLE 8.—*Standardized stomach-cancer mortality ratios (SMR)* for metropolitan and nonmetropolitan counties in 8 geographic areas, by sex—United States, whites, 1949–51*

Geographic area (*geographic division approximated*)	White males			White females		
	Metropolitan counties	Nonmetropolitan counties	Metropolitan/Nonmetropolitan	Metropolitan counties	Nonmetropolitan counties	Metropolitan/Nonmetropolitan
1 (Northeast)	153	123	1. 24	83	67	1. 24
2 (South Atlantic)	102	90	1. 13	52	52	1. 00
3 (East South Central)	93	100	. 93	51	63	. 81
4 (West South Central)	97	104	. 93	51	62	. 82
5 (East North Central)	141	124	1. 14	73	69	1. 06
6 (West North Central)	118	126	. 94	61	72	. 85
7 (Mountain)	107	134	. 80	61	72	. 85
8 (Pacific)	126	120	1. 05	62	54	1. 15

*Standardized mortality ratios are expressed as percentages of the total United States rate (both sexes, all races) for stomach cancer, after adjustment by the indirect method for age.



TEXT-FIGURE 6.—Age-specific mortality rates per 100,000 population from stomach cancer for metropolitan (with central city) and nonmetropolitan counties, by sex. United States, whites, 1949–51.

of a factor introduced into the urban or rural environment affecting only the younger cohorts, or reflect the influence of competitive mortality from other causes at older ages, or of migration of elderly, ill people from town to country, is unknown. The first alternative seems improbable since this would lead, over a period, to larger urban-rural differences. Accurate information on trends in urban and rural rates in the United States is not available. The 1940 cancer mortality data reported by Spiegelman (*40*), the only earlier source of information on urban and

rural residents, dealt with the total digestive system and gave no further details on the stomach. However, since the urban-rural ratio of rates for the total digestive system was higher in 1940 than in 1950, it seems more likely that the ratio for stomach cancer would have been reduced rather than increased in the subsequent decade. Stocks (*29*) has reported little change in the relationship for stomach-cancer mortality between large towns and rural districts in England and Wales from 1920 to 1951.

The relatively small urban-rural disparity in the United States is duplicated elsewhere. Stocks (*39*) has noted that in England and Wales the urban-rural gradient is smaller for the stomach site than for deaths from all sites. The Norwegian mortality data for stomach cancer show minor urban-rural differences (*6*). In Denmark the most recent incidence rates (1948–52) are slightly lower in Copenhagen and its suburbs than in provincial towns and rural areas (*41*). The first reports from the Finnish cancer register do not demonstrate any consistent urban excess (*9*). Olinder (*42*) has reported stomach-cancer mortality to be higher in Swedish country districts than in cities. The experience of these European countries, where population mobility is less than in the United States, suggests that further exploration of American data, with a classification based on complete residence histories rather than place of residence at time of diagnosis or death, might not be rewarding.

Regional Variation

The variation of stomach-cancer mortality by areas within a country has been investigated most extensively in England and Wales, the Netherlands, and Canada (*19, 28, 39, 43*). Work on this variation has been hampered in the United States by the lack of age detail for specific sites in cancer mortality presentations for states and smaller subdivisions. These deficiencies have now been partially remedied with special tabulations of the 1949–51 mortality data.

Table 9 presents death rates age adjusted by the direct method for the total digestive system and the major component sites. The 9 geographic divisions (groups of states) have been ranked by order of magnitude of the rates for the total digestive system. The digestive system accounts for a sizable part of the variation, among divisions, in cancer mortality for all sites. Stomach-cancer mortality is definitely higher in the Northeast and North Central parts of the country than in the South, and this relationship holds also for the intestines, rectum, and esophagus. The regional variation for stomach cancer is not accompanied by compensatory differences for other digestive sites. Rather, stomach cancer fits into the larger pattern of variation for the total digestive system. As a matter of fact, the spread of 55 percent between the high and low regional rates for stomach cancer is smaller than that noted for the intestines, rectum, or esophagus.

The regional distributions of the age-specific rates (table 10) generally conform to that presented by the age-adjusted rates, but the relative

TABLE 9.—*Age-adjusted* mortality rates per 100,000 population from cancer of all sites and of selected sites in the digestive system, by sex, and by geographic division—United States, whites, 1949–51*

Division†	All sites‡ (*140–205*)	Digestive system, total (*150–156.1, 157–159*)	Stomach (*151*)	Intestines (*152, 153*)	Rectum (*154*)	Esophagus (*150*)	Pancreas (*157*)	Biliary passages and liver (*156.1*)
			White males					
Middle Atlantic	172.0	75.6	23.7	19.0	11.9	5.4	8.2	6.0
New England	167.0	74.0	22.6	19.6	11.3	5.7	8.0	5.7
East North Central	151.1	65.5	21.6	16.1	8.8	4.5	7.1	6.5
Pacific	144.1	58.0	20.0	12.9	7.8	3.4	8.0	4.9
West North Central	135.4	57.3	20.5	14.0	6.8	2.8	6.9	5.5
Mountain	125.1	51.3	21.0	10.2	5.3	1.7	6.7	5.7
South Atlantic	130.2	47.8	15.6	10.9	5.2	2.9	6.8	5.6
West South Central	127.4	46.7	16.5	9.6	3.9	2.1	7.0	6.7
East South Central	119.7	44.3	15.3	10.5	3.7	2.1	5.7	6.1
			White females					
Middle Atlantic	147.8	56.7	13.2	20.6	7.7	1.3	5.4	7.2
New England	144.1	54.2	12.2	20.9	7.3	1.1	5.3	6.4
East North Central	138.0	49.8	11.5	17.2	6.0	0.9	4.9	8.4
West North Central	124.1	44.4	11.0	15.6	4.7	0.8	4.4	7.2
Pacific	127.8	41.7	9.7	14.1	5.6	1.0	4.9	5.5
Mountain	114.9	39.9	11.0	12.1	4.2	0.6	4.3	6.7
East South Central	117.0	38.6	9.5	12.9	3.4	0.9	3.7	7.3
South Atlantic	119.7	38.2	8.5	13.1	4.3	1.0	4.3	6.0
West South Central	116.3	36.7	9.2	11.9	3.2	1.1	4.0	6.5

*Rates adjusted to age distribution of the United States 1950 census population.

†As defined by U. S. Bureau of the Census.

‡International List Numbers, sixth revision, in parentheses.

TABLE 10.—*Age-specific mortality rates per 100,000 population from stomach cancer, by sex and by geographic division—United States, whites, 1949–51*

Division*	Age-adjusted rate	25–34 (*yrs.*)	35–44 (*yrs.*)	45–54 (*yrs.*)	55–64 (*yrs.*)	65–74 (*yrs.*)	75–84 (*yrs.*)	85 and over (*yrs.*)
	White males							
Middle Atlantic	23. 7	0. 8	4. 9	21. 0	67. 3	152. 4	234. 2	245. 5
New England	22. 6	1. 0	4. 8	20. 5	62. 8	142. 2	226. 0	253. 9
East North Central	21. 6	0. 5	4. 0	16. 8	56. 9	136. 7	241. 5	293. 0
Mountain	21. 0	0. 4	3. 8	15. 9	58. 8	135. 9	227. 1	222. 4
West North Central	20. 5	0. 6	3. 5	14. 9	46. 4	131. 6	255. 8	307. 7
Pacific	20. 0	0. 7	3. 4	15. 5	53. 2	123. 8	224. 0	297. 2
West South Central	16. 5	0. 7	4. 2	13. 9	42. 2	98. 3	183. 2	239. 3
South Atlantic	15. 6	0. 5	3. 7	14. 0	40. 7	91. 4	172. 1	229. 8
East South Central	15. 3	1. 1	4. 4	12. 8	35. 7	91. 2	185. 3	207. 0
	White females							
Middle Atlantic	13. 2	0. 8	3. 0	11. 2	32. 8	80. 0	150. 0	176. 7
New England	12. 2	0. 6	3. 7	10. 4	30. 6	70. 1	141. 5	185. 6
East North Central	11. 5	0. 7	2. 5	8. 1	27. 3	66. 0	147. 0	206. 8
Mountain	11. 0	1. 1	2. 1	7. 6	26. 0	61. 2	149. 6	189. 1
West North Central	11. 0	0. 6	2. 1	7. 3	22. 5	63. 4	151. 6	233. 0
Pacific	9. 7	0. 5	2. 8	8. 1	23. 7	53. 0	119. 2	166. 5
West South Central	9. 2	0. 6	2. 8	7. 6	22. 3	51. 3	116. 5	130. 6
South Atlantic	8. 5	0. 7	2. 0	6. 8	18. 8	50. 2	105. 8	151. 1
East South Central	9. 5	0. 5	2. 2	6. 7	19. 1	57. 8	125. 1	192. 4

*As defined by U. S. Bureau of the Census.

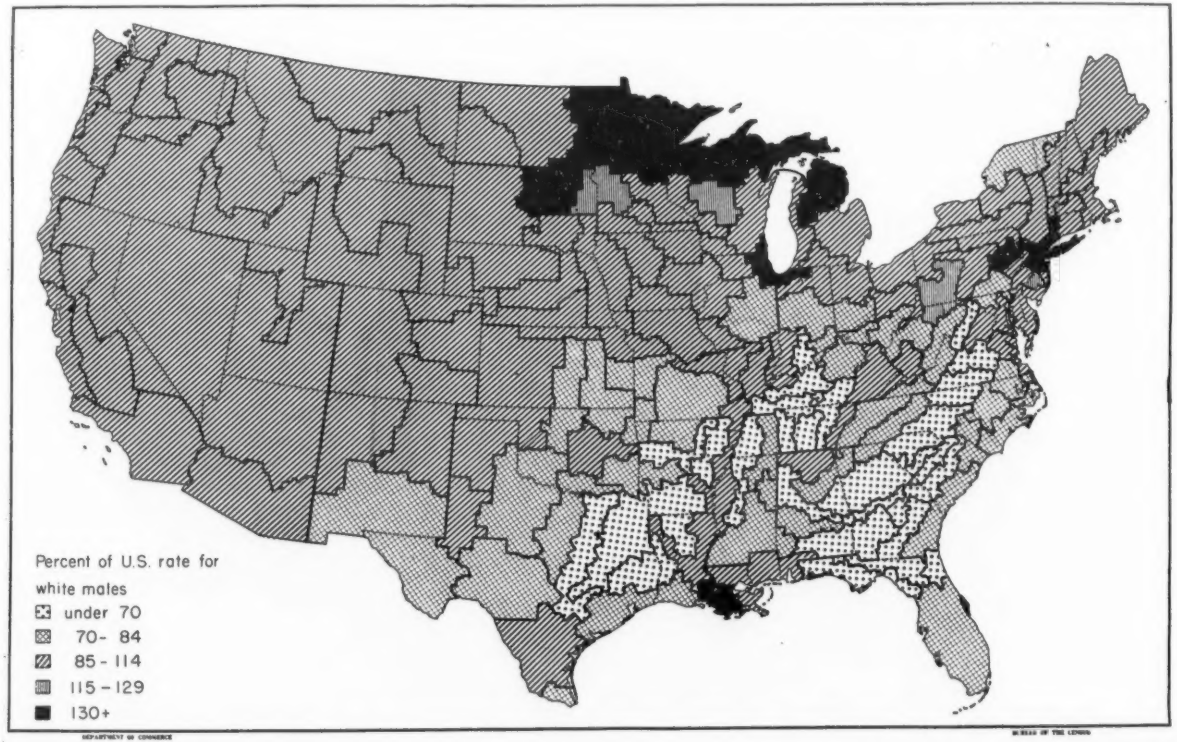
difference between southern and northern states narrows after age 75. White males and females present the same regional patterns.

Recent tabulations of mortality for residents of the 119 economic subregions defined by the United States Bureau of the Census, *i. e.*, groupings of counties that cross state lines, pursue the study of geographic variation into still smaller areas. Standardized mortality ratios were computed for each subregion for the years 1949–51 by the indirect method of relating the known deaths to the number expected if the corresponding national experience had prevailed in the subregion. Results were computed separately for the 4 race-sex groups. The data for white males and females are presented in text-figures 7 to 12 for the subregions and their metropolitan and nonmetropolitan county components. Because of the concentration of nonwhites in the South and the recent migration of Negroes to the North, it is not worthwhile to present the data for nonwhites. The study of geographic variation has not yet been extended to such detailed subdivisions as individual counties. For this purpose an observation period longer than 3 years would be required. The punch cards for cancer deaths which identify county of residence have been preserved and it would be possible to investigate later the pattern for individual counties.

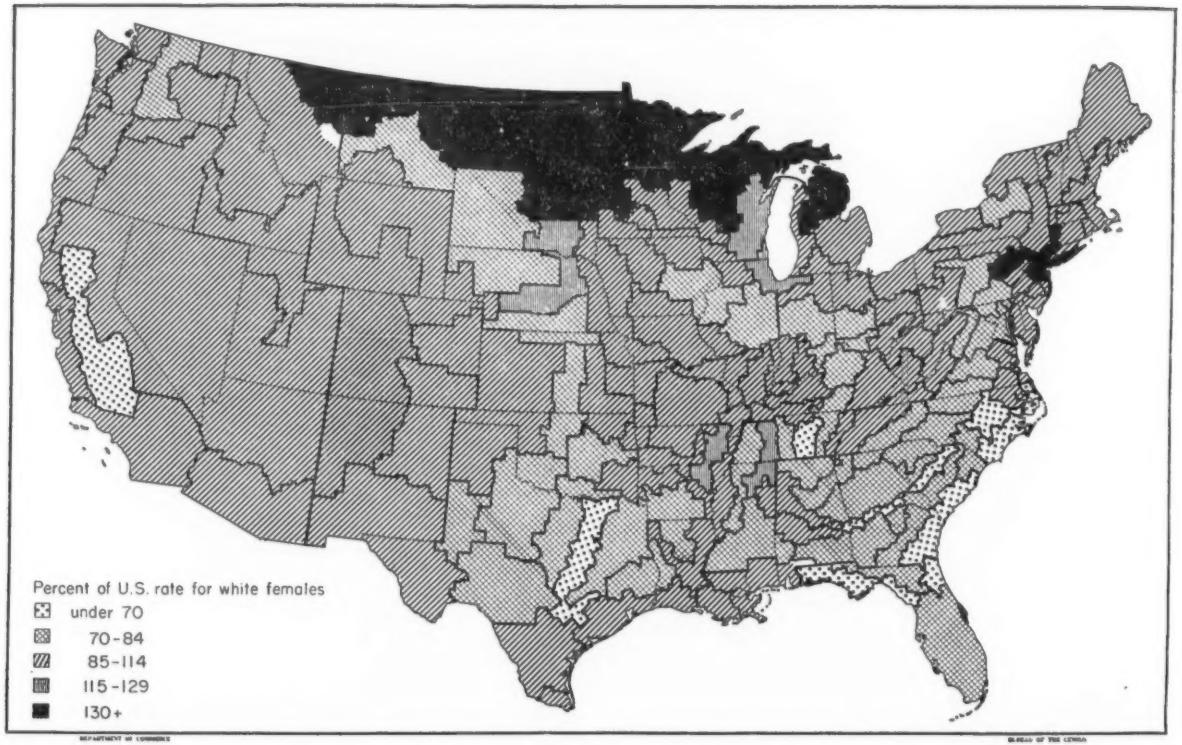
The range in SMR's for subregions is quite large, the highest values being about 3 times as large as the lowest ones observed. Even though 3 years' experience has been used, the results for an individual subregion, especially the less populous ones, are subject to sampling variation. Results for any one region should be interpreted with caution, although some are suggestive enough to warrant further study, and primary attention should be directed to the regional configurations.

The maps dealing with the combined metropolitan and nonmetropolitan experience show a belt of subregions with low ratios in the South from Virginia to Texas. Regions with the largest cities (New York, Chicago, *etc.*) have high SMR's. Since the latter contain a large proportion of the United States population, many of the remaining regions must show low SMR's when compared with the national experience. It is interesting to observe, however, that the highest SMR's are not in the largest cities but in an area across the northern tier of states from North Dakota to Michigan. When the results for metropolitan counties alone are examined, subregion 66 in Minnesota, Wisconsin, and Michigan has a high SMR, exceeding that for most big city subregions. The concentration of low rates in the South persists in the metropolitan county presentation.

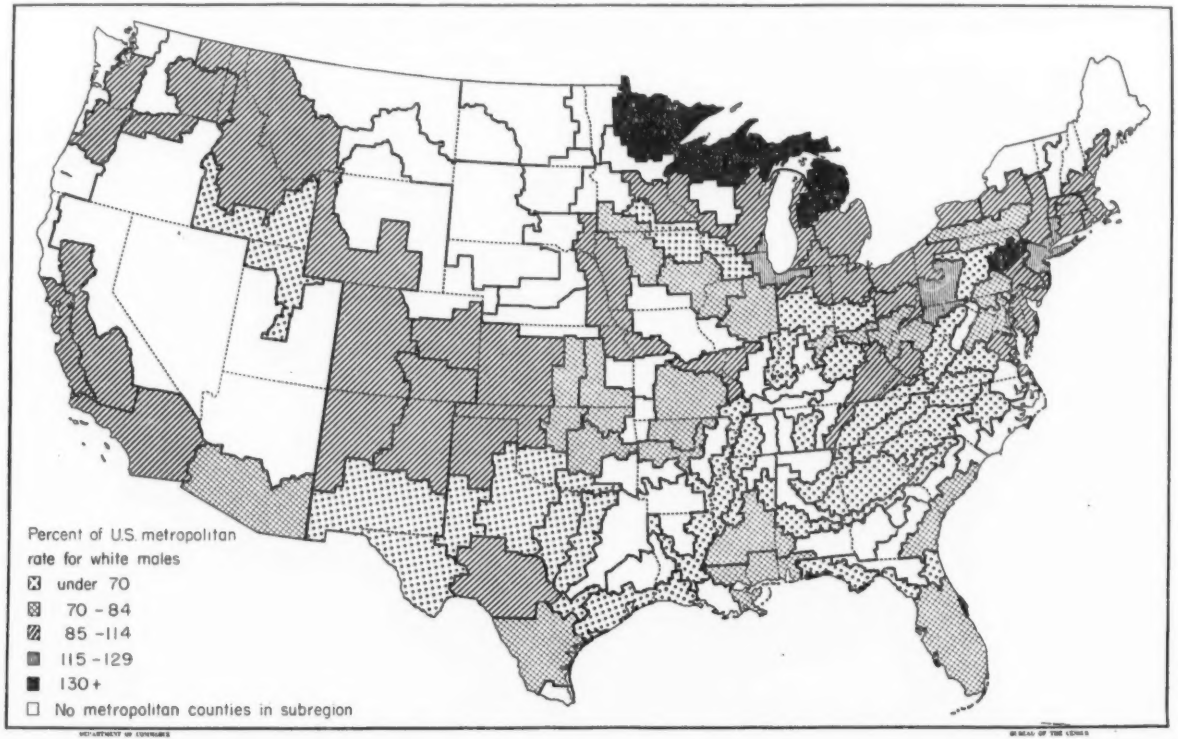
The northern subregions, particularly those with major population centers, have large foreign-born populations, a situation unlike the South where most of the population is native-born of native parentage. Part of the difference between the North and South can probably be accounted for by higher stomach-cancer rates among the foreign-born. This conjecture is supported by the scanty available evidence discussed in the section on nativity. When the analysis of 1950 cancer mortality by country of birth is completed, it should be possible to estimate how much of the North-South difference would remain if the comparison were restricted to the native-born population.



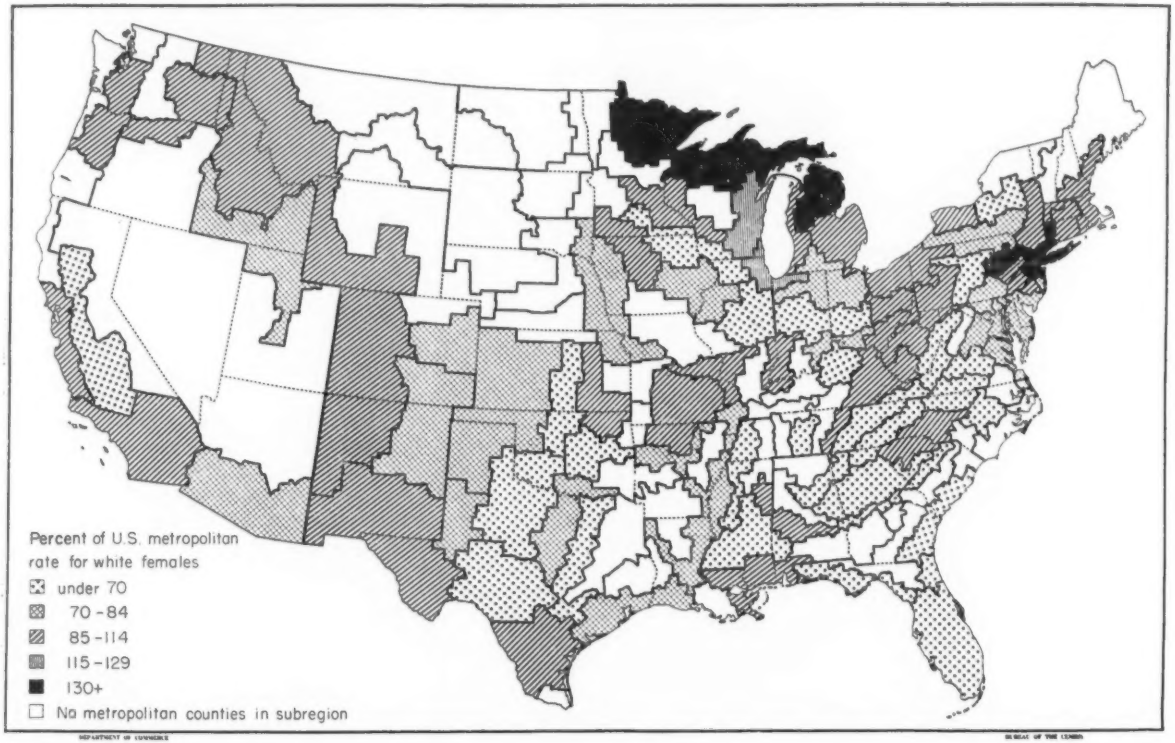
TEXT-FIGURE 7.—Standardized stomach-cancer mortality ratios (SMR) by economic subregion. United States, white *males*, 1949–51.



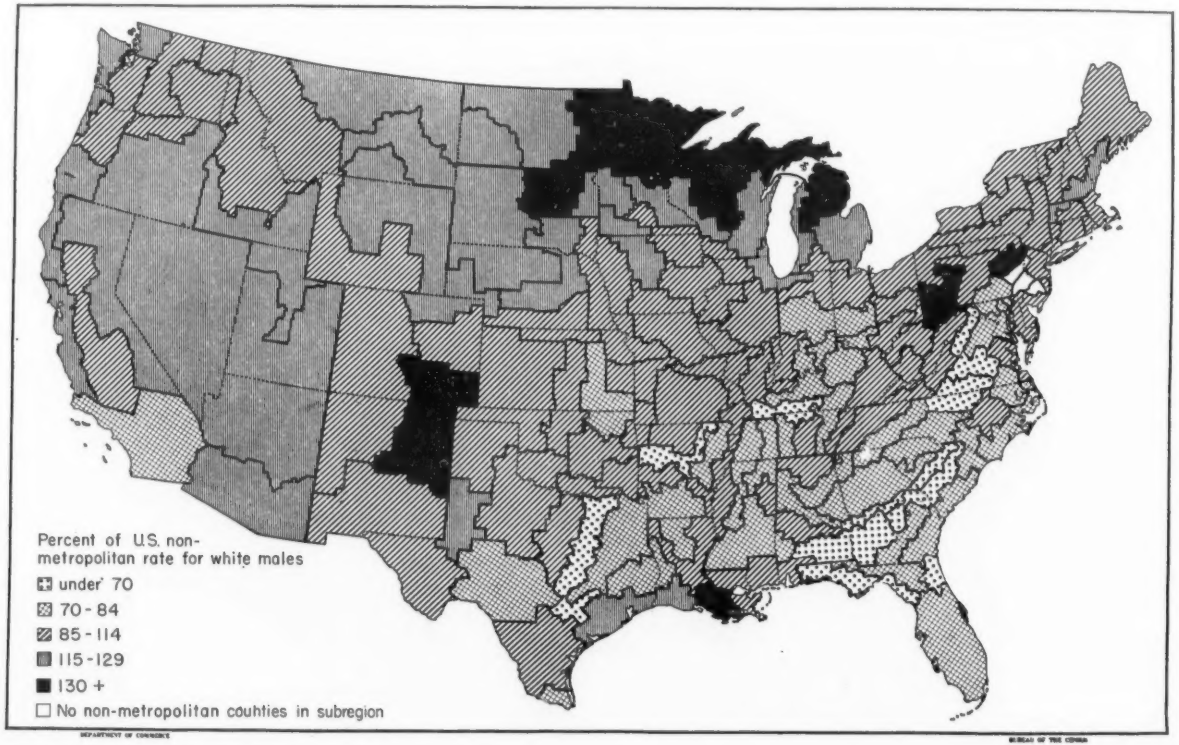
TEXT-FIGURE 8.—Standardized stomach-cancer mortality ratios (SMR) by economic subregion. United States, white *females*, 1949–51.



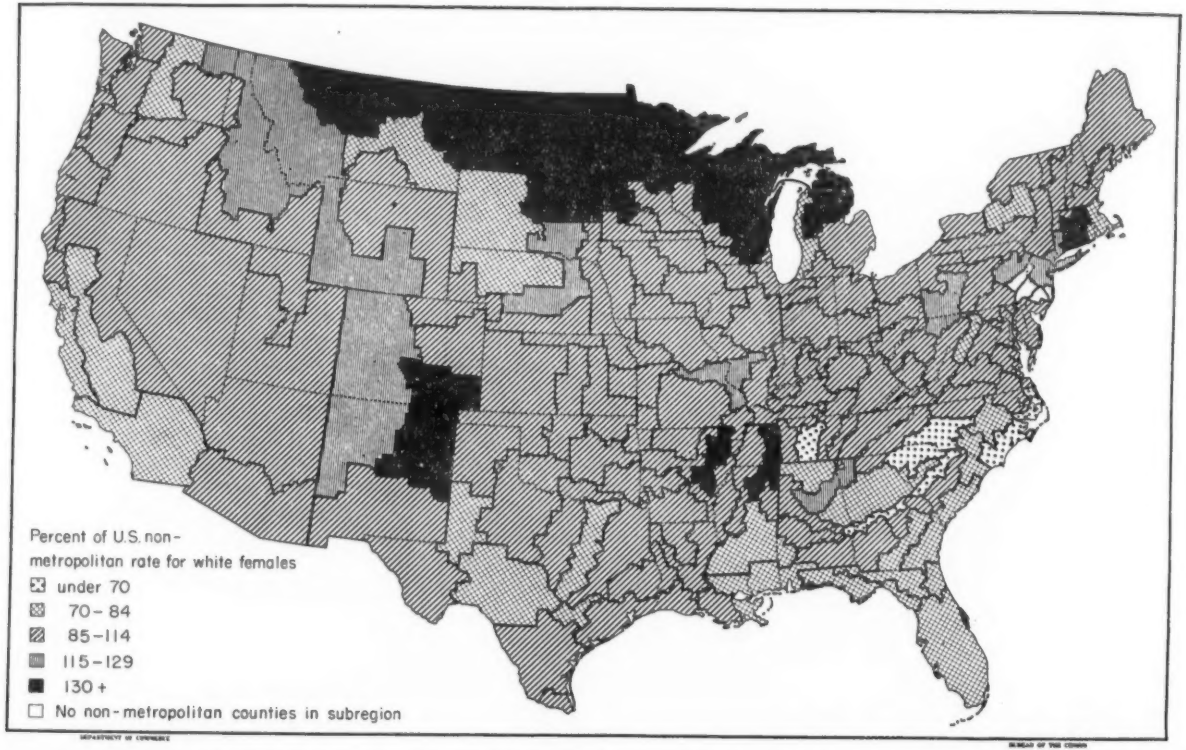
TEXT-FIGURE 9.—Standardized stomach-cancer mortality ratios (SMR) by economic subregion, for metropolitan counties. United States, white *males*, 1949–51.



TEXT-FIGURE 10.—Standardized stomach-cancer mortality ratios (SMR) by economic subregion, for metropolitan counties. United States, white *females*, 1949–51.



TEXT-FIGURE 11.—Standardized stomach-cancer mortality ratios (SMR) by economic subregion, for nonmetropolitan counties. United States, white *males*, 1949–51.



TEXT-FIGURE 12.—Standardized stomach-cancer mortality ratios (SMR) by economic subregion, for nonmetropolitan counties. United States, white *females*, 1949–51.

The results for nonmetropolitan counties in text-figures 11 and 12, which eliminate large cities and their foreign-born groups and hence may be less influenced by variation associated with nativity, suggest that a North-South difference would still be present. The low SMR's appear in the same regions from Virginia to Texas, while the area of high SMR's along the Canadian border is even more striking. The difference between North and South may be of long standing. Stocks (*44*) computed the proportion of stomach-cancer deaths in relation to all cancer deaths for 1900 and found the lowest relative frequencies in the southern states.

The 10-city morbidity surveys also revealed that the northern cities had a higher stomach-cancer incidence in both 1937 and 1947 (table 11). Since differences attributable to diagnostic factors should be minimized in comparisons of areas served by university centers with good medical facilities, this would argue against attributing regional differences in mortality solely to differences in diagnosis.

TABLE 11.—*Age-adjusted* incidence rates per 100,000 population from stomach cancer—United States, 10 cities, 1937 and 1947*

Cities	1947	1937	Percent change
Ten cities	26.4	34.7	−23.9
North	27.3	37.9	−38.0
Chicago	28.5	41.4	−31.2
Detroit	27.6	28.8	−4.2
Philadelphia	25.9	32.3	−19.8
Pittsburgh	25.1	38.4	−34.6
South	20.2	22.0	−8.2
Atlanta	14.9	16.1	−7.5
Birmingham	19.1	13.3	43.6
Dallas	16.3	17.6	−7.4
New Orleans	30.5	37.1	−17.8
West	28.0	29.3	−4.4
Denver	25.1	22.6	11.1
San Francisco	28.8	31.0	−7.1

*Rate adjusted to age distribution of the United States 1950 census population.

The variation in stomach-cancer mortality by latitude within the United States seems consistent with the finding that countries farthest removed from the equator have the highest rates. Curiously enough, a similar effect on a more compressed geographical scale has been noted in England. According to Stocks (*39*), the northern counties of Wales had higher rates (1921–30) than the central counties. He also examined (*43*) stomach-cancer mortality in the large towns of England and Wales for the years 1921–39 and found the 83 county boroughs to show great differences ". . . which cannot be explained either by chance variations or by differing accuracy of certification of the cause of death" and that the standardized mortality ratios tended to be higher in the northern towns.

The British and Dutch experience on the magnitude of regional variation is in accord with the American results, suggesting that regional

variations attributable to reasons other than ethnic group differences do exist. In England the highest area rates for stomach cancer exceeded the lowest observed by more than 100 percent (*43*). Tromp (*28*) found, for males over 50 years of age, that the highest Netherlands provincial rate was about double the lowest one. On the other hand, variation in the Canadian data probably reflect an ethnic group influence. The provinces with the highest proportion of French Canadians (Quebec and New Brunswick) have higher stomach-cancer mortality than the other provinces with the exception of Nova Scotia (*19*). This would tie in with the observation of Lombard and Doering (*38*) that French Canadians in Boston had high death rates from this cause.

Some rather striking associations with soil type have been reported by several authors. Tromp and Diehl (*45*) have observed that persons residing on peat soils in the Netherlands had higher stomach-cancer mortality than those on sandy or river clay soils. [illegible]ales, Legon (*46*) found low mortality similarly associated with an absence of peat in the soil. Davies and Griffith (*47*) were able to associate stomach cancer with soil series in Anglesey, independent of water supply and social class; people living on soils characterized by great biological activity over a long season and by a high content of organic matter had the highest mortality. None of the reports discussed the agricultural uses of these soils. The United States subregions are large and embrace a variety of soil conditions so that the observed variation in mortality would need to be related to other factors in addition to soil type. One cannot be certain that similar relationships for soil type could even be demonstrated for small areas within the United States, if the effects are due to the distribution of trace elements in food as Tromp and Diehl suggest, because high population mobility and the national character of the food distribution system might have diluted beyond detection effects linked with soil types.

Nativity

Lombard and Doering (*38*) have provided the only direct measures of variation in stomach-cancer mortality among the foreign-born in the United States. Thirty years ago they found that the foreign-born in Boston had a higher mortality for this site than the native-born. Their data also suggested differences by country of birth, with high rates appearing for Poles, Russians, and French Canadians and low rates for Italians. Lombard and Doering at that time were inclined to interpret the differences by ethnic group for all sites as a general effect connected with socioeconomic status. The possibility remains, however, that there are specific relationships between country of birth and stomach cancer which are not reproduced for other cancer sites. If this should be proved, one might profitably examine the prevailing customs among the foreign-born groups for leads on possible etiologic factors.

No further analyses of stomach-cancer mortality by country of birth of decedent have been completed since the report by Lombard and Doering. Work now in progress to add nativity data to the punch cards for

the 1950 cancer deaths in the United States should cast more light on this topic. Besides the indirect evidence of nativity effects provided by the patterns of urban-rural and regional variation already discussed in this paper, there are other findings which tend to support the early Lombard-Doering results. Results from the New York State cancer register suggest that stomach-cancer incidence is high in Buffalo, a city with a large Polish population, and relatively low in Rochester where Italians are the dominant foreign-born group (*13*). In this connection, a retrospective study of patients with stomach cancer and of controls, matched with respect to occupation and other factors, admitted to Roswell Park Memorial Institute in Buffalo, revealed an excess number of persons of Polish birth among the diagnosed cases (*48*). Patno (*49*), in analyzing cancer experience in Pittsburgh, found the lowest prevalence and mortality rates for cancer of the digestive organs in an area predominantly Italian.

Social Class

Measurement of the variation in cancer risk by social class has been approached in two ways. One method relies on classifying and consolidating geographic areas within cities, using as an index for this purpose average family income or housing rental. The other method uses data on occupation for socioeconomic grouping.

The most extensive American data on this point have been collected in the 10-city cancer morbidity surveys. Dorn and Cutler (*50*) allocated newly diagnosed cases to census tracts and used rental data to array the tracts for each city into 5 classes, each containing approximately the same number of people. Their results, summarized in table 12, indicated that white men and women in the lowest social class had a stomach-cancer incidence more than 50 percent in excess of that for persons in the highest

TABLE 12.—*Summary of standardized ratios* of stomach-cancer risk by social class*

Social class†	United States, 10 cities, newly diagnosed cases, 1947 Dorn and Cutler (*50*)		Copenhagen, newly diagnosed cases, 1943–47 Clemmesen and Nielsen (*52*)		England and Wales, deaths, 20–64 years, 1950 Logan (*56*)	
	White males	White females	Males	Females	Males	Married females
All classes	100	100	100	100	100	100
1 (high)	71	80	87	76	57	57
2	90	98	89	101	67	72
3	102	98	89	93	100	101
4	105	112	119	104	114	106
5 (low)	124	112	108	107	132	138

*Adjusted for age.

†For United States, 10 cities, and Copenhagen, housing rentals were used as an index for grouping geographic areas into social classes; for England and Wales, occupations of husband were grouped into 5 classes from "professional" to "unskilled."

class; stomach-cancer data for nonwhites revealed similar gradients. The same source material showed the esophagus had a social class gradient resembling that for the stomach, while the intestine exhibited almost no variation in this respect. Since the effects were specific for individual sites, it seems unlikely that differences in diagnostic and medical care facilities could have accounted for the social class variation noted by these authors for stomach cancer.

Cohart (*51*) observed a similar relationship when he investigated the socioeconomic distribution of gastric cancer in New Haven, Connecticut. Additional information may become available when analysis of the United States mortality data for 1950 by occupation, industry, and social class is completed.

In Denmark, Clemmesen and Nielsen (*52*) used rentals to group districts into 5 classes within Copenhagen. They also observed an association between social class and stomach-cancer incidence, though the magnitude of the excess in the lowest class was not as large as in the American experience (table 12). Their results for the esophagus and the colon also coincided with those obtained by Dorn and Cutler. A similar association turned up in a Norwegian study. Rennaes and Østberg (*53*) found the Oslo parishes with the lowest average rentals had experienced substantially higher stomach-cancer mortality during the years 1930–50. More recent reports from Oslo, which have given particular attention to the question of diagnostic errors, reconfirm the social class differential (*54*, *55*).

The Registrar General of England and Wales has, for more than 30 years, reported on socioeconomic differentials in mortality and has used for this purpose occupational data given on the death certificate. Stomach-cancer mortality among males in that country has always exhibited a strong inverse relationship with social class. The 1950 data, as reported by Logan (*56*), appear in table 12. When married women are classified by their husband's occupation, similar gradients are found; wives of unskilled workers have rates more than double those for wives of professional men, which suggests that domestic, nonoccupational factors are involved. The range between the extreme classes is larger in the British data than reported elsewhere. The British mortality data have not generally pinpointed specific occupational exposures as being associated with excess stomach-cancer risks, and the fact that the effects also appear for spouses supports the interpretation that the effects are associated with characteristics common to many occupations at the same socioeconomic level. Young and Russell (*57*) found an indication of an excess of stomach-cancer deaths among iron, coal, and slate miners, and a similar result has recently been obtained by Kraus *et al.* (*48*) who, in a controlled study of diagnosed cases, found a significantly higher percentage with a history of prolonged exposure to iron dust.

When one considers the variety of source materials and study approaches, the results from various countries agree, surprisingly, on the magnitude of differences associated with social class.

Endogenous Factors

The evidence in regard to genetic factors in stomach cancer in man comes principally from retrospective studies of cancer experience among relatives of patients and matched controls. The associations with blood group, an inherited characteristic, the excess incidence among patients with pernicious anemia and achlorhydria, and the pedigrees for isolated "cancer families" may be regarded as consistent with, and lending support to, the findings of the statistical studies in retrospect.

Videbaek and Mosbech (*58*) found that the incidence of stomach cancer was 4 times greater among the relatives of 302 cancer patients when compared with the relatives of a similar number of controls. They also reported pernicious anemia to be twice as frequent among relatives of patients with the disease. Macklin (*59*) investigated 800 families and found children and siblings of stomach-cancer patients had experienced over 4 times the expected number of stomach-cancer cases, a result in numerical agreement with that of Videbaek. While Macklin points out that only 10 percent of the families investigated were those of patients with gastrointestinal cancer, her tables do not segregate the data for this 10 percent. Macklin (*60*) has also interpreted some results of Hanhart (*61*) on the frequency of cases among the offspring of 121 couples, both of whom had cancer, as indicating an excess stomach-cancer risk in some families.

Woolf (*62*) traced the death certificates of parents and siblings of 200 gastric-cancer patients who died in Utah between 1940 and 1950. Forty-four deaths from gastric cancer were revealed in this group as contrasted to 25 deaths expected on the basis of the mortality rates for the total Utah population of comparable age. The 76 percent excess is substantially smaller than that noted in Videbaek's and Macklin's studies. Woolf also checked death certificates for the relatives of 186 control propositi and found close agreement in the known and expected numbers of gastric-cancer deaths. He found no excess mortality from this cause among 540 spouses of persons who died from gastric cancer.

State *et al.* (*63*) and Hagy (*64*) have also reported higher frequencies of stomach cancer among relatives of patients as compared to relatives of controls, though their individual results were not statistically significant.

Because retrospective investigations of families are important in studies of human genetics, a brief mention of a source of bias in such material, and not discussed by any of the authors cited, is warranted. The bias is well known in other fields under "the problem of the index case." For example, it has long been recognized that data on family size (number of children) obtained from siblings, rather than parents, are biased. The reason is that 2 or 3 potential "index cases" are present in the population for 2- and 3-child families as opposed to one for 1-child families and none for childless couples. The translation to stomach-cancer data is obvious. Do *families* with 2 or 3 cancer patients have double or triple the probability of being represented in source material and thus having a representative selected as a propositus than families with only 1 patient?

While it may be possible to formulate rules for selecting "index cases" which will minimize the bias, all the studies cited except that of Hanhart appear vulnerable on this point.

Eliminating the propositus from computations on cancer risk, which is the usual analytical procedure, does not compensate for this bias. Greenwood and Yule (*65*) have discussed the method for adjusting the bias in connection with studies of family size and order of birth. An appropriate correction consists in adjusting the frequency distribution for the number of cases per family through division by the corresponding number of potential "index cases." An application to the proband data reported by Videbaek (*58*) is illustrated in table 13.[4]

Much of the difference between Videbaek's proband and control families could be accounted for in this fashion. On the approximately correct assumption that the proband and control families have the same distribution with respect to family size, the reduced excess of stomach-cancer cases among relatives of probands may be estimated to be about 60 percent:

Estimated number of cases				
100 proband families		100 control families		
$80.7 \times 0 =$	0	$86.9 \times 0 =$	0	
$15.6 \times 1 =$	15.6	$11.3 \times 1 =$	11.3	$24.3/14.9 = 1.63$
$2.6 \times 2 =$	5.2	$1.8 \times 2 =$	3.6	
$0.9 \times 3 =$	2.7			
$0.2 \times 4 =$	0.8			
Total	24.3	Total	14.9	

Since Videbaek does not state explicitly his procedures for selecting probands, it is not possible to venture an opinion on the degree to which this bias does affect his data. Some of his other results [table 4, (*58*)], the low ratio in the control series of observed-to-expected stomach-cancer cases in the presence of close agreement of known and expected numbers for other sites, support the conjecture that there is bias. If families with several cases are overrepresented in the proband series, the opportunity exists for underrepresentation of such families in a control series, particularly if the proband families account for a sizable proportion of all the cases within the study area.

While the degree of familial association may be smaller than indicated by these retrospective studies, the comments should not be interpreted as denying the existence of a familial tendency to stomach-cancer susceptibility. Prospective studies, where individuals are classified in advance and their subsequent histories and those of their sibs and descendants traced, are not subject to the index case bias. Fragmentary insurance data of the latter type point to a modest excess gastric-cancer risk in some families. Dublin and Marks (*66*) found that insured persons with a family history of gastric cancer had a subsequent gastric-cancer mortality

[4] It may also be shown that the index case bias would reduce slightly the representation of stomach-cancer cases among families of controls. In the extreme situation where every family member had stomach cancer, the probability of that family appearing in the control series would be nil.

TABLE 13.—*Application of proband data reported by Videbaek*

Number of cases in family (*excluding proband and control*)	Distribution of families of probands as reported by Videbaek and Mosbeech (*58*)		Adjusted distribution of families of probands, following method of Greenwood and Yule		Percent distribution of families of controls, as reported by Videbaek and Mosbeech (*58*)
	Number	Percent	Estimated number (*reported number÷X**)	Estimated percent	
Total families	302	100.0	–	100.0	100.0
0	196	64.9	196.0	80.7	86.9
1	76	25.2	38.0	15.6	11.3
2	19	6.3	6.3	2.6	1.8
3	9	3.0	2.3	0.9	–
4	2	0.7	0.4	0.2	–

*X=number of cases in family, including proband

about one third greater than other insured persons, though their result was of borderline statistical significance.

More prospective studies on the stomach-cancer experience of sibs and children of stomach-cancer patients are needed to establish more firmly the magnitude of the effects associated with the family. Parenthetically it might be noted that the clustering of gastric-cancer cases by family is a necessary but not sufficient condition for establishing a genetic factor, since environmental factors common to a family, such as the selection and preparation of foods, might in theory produce the same effect. For this reason such studies should incorporate findings on spouses like those studies reported by Woolf. Mider (*67*) has pointed out the possibilities for forward studies within the United States by utilizing data accumulated in the cancer registers maintained by several state health departments. If the low incidence in the United States should prove attributable to less exposure to, or greater protection conferred by, environmental factors, studies here on family distribution would have the added advantage of being less confounded by nongenetic factors.

The observations by Videbaek on the excess of pernicious anemia cases among relatives of gastric-cancer patients are not influenced by the index case bias and may be accepted at face value. Studies with pernicious anemia patients as the point of departure corroborate this result. Hitchcock *et al.* (*68*) reporting on the excess stomach-cancer risk among achlorhydric patients found that 71 pernicious anemia patients developed an excess number of gastric-cancer cases. Berkson (*69*) obtained similar results in his forward study of persons with pernicious anemia and achlorhydria. Doehring (*70*) also noted that his pernicious anemia patients, those hospitalized and those autopsied, had an excess of gastric-cancer diagnoses. Doehring's results agreed with other reports in the literature summarized by him.

Several papers have reported an excess frequency of blood group A in gastric-cancer patients among predominantly white populations (*71–74*), though Speiser (*75*) recently found no association in his Viennese series of diagnosed cases and controls. A small but significant excess of group A individuals among gastric-cancer patients in Japan has also been reported by Segi *et al.* (*76*). Similar associations between blood group and cancer of the colon and rectum have not been demonstrated (*77*). Blood group data for cancer patients and controls permit estimates of the relative risk among group A individuals as compared to a unit risk for the remainder of the population, following a procedure outlined by Cornfield (*78*); these estimates from 4 series are given below:

	Relative risk for persons of blood group A.
Koster (*74*)	1.34
Aird (*71*)	1.23
Buckwalter (*73*)	1.19
Billington (*72*)	1.07

The sampling variation of these estimates of relative risks is fairly large, and the data of Koster, Aird, and Buckwalter are not inconsistent. Billing-

ton, in subdividing his data by anatomical location, found prepyloric and cardiac lesions significantly associated with blood group A and fundus lesions with group O. Other reports have linked an excess of cardiac lesions with group A.

While the weight of the evidence favors an excess risk among persons of blood group A (if it were shown that several negative results had not been published, this conclusion could be challenged), this factor, even if established unequivocally, could scarcely account for the international variation in stomach-cancer mortality. From the estimated scale of relative risks, an absolute difference of as much as 15 percent in the national percentage of persons of blood group A would be required to achieve a 3 to 5 percent difference in incidence and/or mortality. Furthermore, blood group distributions are too stable over a time to be advanced as an explanation for the recent downtrend in stomach-cancer mortality noted in certain countries. The effects associated with blood group, cancer families, and other biological factors can undoubtedly be found in all countries, but in comparisons of countries and smaller population aggregates, these effects are diluted by mixture with populations not affected by these factors. Consequently, these effects, while in themselves important, do not appear large enough to account for many of the epidemiologic characteristics observed for stomach cancer.

Other Factors

Other factors that have been discussed in relation to stomach cancer include tobacco and alcohol, marital status, and laxatives.

There is conflicting evidence on the association of stomach cancer with tobacco. Doll (*1*) has referred to an unpublished British study of patients and controls which he conducted with Hill, and which revealed no association with smoking history. Segi (*76*) in a similar study of Japanese males found a small excess of smokers among cancer patients. More extensive American data, presented by Hammond and Horn (*79*) and covering 4 years' follow-up of almost 200,000 males, showed a 61 percent excess of stomach-cancer deaths among men with a history of cigarette smoking compared to nonsmokers. In the latter study the result for the stomach was an intermediate to a very large excess for the esophagus, among smokers, and there was no difference between cigarette smokers and nonsmokers for the colon and rectum. More evidence is needed. In view of the widespread distribution of the cigarette smoking habit in the United States (*80*), this factor cannot be related to the regional differences which have been noted for stomach cancer. It might have some bearing on the sex ratio since this habit is far more prevalent among men.

Alcohol consumption is a difficult topic to investigate because of the problems in securing reliable histories. The British occupational mortality data (*81*) suggest that alcohol consumption is not an important factor since the data fail to demonstrate an excess risk for bartenders and other persons in the trade who, as a class, are known to use alcoholic

beverages consistently. Segi (*76*) in a controlled retrospective study of Japanese males has observed an association between stomach cancer and habitual use of sake, a rice wine.

An association of stomach cancer with marital status seems doubtful. Cancer mortality data for the total digestive system (separate data are not available for the stomach) are higher for unmarried men and women, but the difference is of the same order of magnitude as that observed for cancer of all sites and for all causes of death (*82*). Specifically, cancer of the digestive system does not follow cancer of the breast and of the female genital organs in exhibiting either an unusually marked excess or deficit of deaths among unmarried persons. The more likely interpretation for the digestive system data is that the results merely reflect nonspecific, selective effects of marriage and not the operation of special environmental factors among unmarried men or women.

Little is known about the effect of laxatives. Boyd and Doll (*83*) in a controlled study of patients with gastrointestinal cancer detected an association for one agent, liquid paraffin, but nothing of significance for other common laxatives. The interpretation of these results is complicated by the reasons for the use of laxatives and by the relationship of premonitory symptoms to subsequent diagnoses. In any event only a small number of persons uses any specific type of laxative, so that this factor could scarcely be important in determining the epidemiologic characteristics of this disease.

Implications for Epidemiologic Studies

The key findings which emerge in review of the available evidence on stomach cancer are as follows:

a) great variation among countries in stomach-cancer rates;
b) rather stable male-female ratios of rates for each country over the entire range of stomach-cancer rates;
c) a low rate in the United States, with the further distinction that the rates for certain foreign-born groups probably exceed those for the native-born population;
d) the recent continuing downward trend in the United States, a development occurring in some, but not all, other countries;
e) the inverse relationship with socioeconomic class, with little evidence of specific occupational effects;
f) the lack of marked urban-rural differentials;
g) the tendency for countries in northern latitudes to have higher rates, and the presence of north-south differences within the United States compatible with the broader international pattern.

In the search for exogenous factors that might influence the geographic distribution of stomach-cancer patients, dietary factors are the most obvious. None of the findings listed contraindicate a possible connection with some item(s) of diet. International, regional, income, and ethnic group variations in diet are a matter of common knowledge.

Great changes in food habits are known to have taken place during the past half century in the United States. The effect of latitude might also be involved, since climate determines the type of crops which can be grown. There are sex differences both in total food intake and in nutritive content of food. On the other hand, great differences in the food habits of urban and rural residents in the same locality would not be anticipated because of dependence on common sources of supply.

The possible dietary effects fall in 3 general categories: *a*) the presence of a carcinogen occurring naturally in food, including such physical properties as food texture; *b*) a carcinogen introduced in food preparation, including such physical properties as food temperature; *c*) the absence of some protective factor. Since the stomach is considered to be a nonexcretory organ, the direct action of a carcinogenic agent on the gastric mucosa must be considered a plausible hypothesis. However, in the present state of knowledge the possibility of an indirect systemic effect cannot be disregarded.

Most of the impressions about the role of diet which appear in the clinical literature are based on evidence derived from geographic and ethnic group variation in stomach-cancer incidence and diet. Since the epidemiologic evidence favors the notion of a stomach-cancer effect linked with some aspect of diet, and clinical impressions offer no strong contraindications, this line of investigation deserves attention. The remainder of the discussion concentrates on this phase of the problem, though it is recognized that other effects may be postulated and that epidemiologic studies might be devised to test them. While there is no guarantee that associations with diet can be revealed, failure after intensive efforts to establish this family of hypotheses would open the way for alternate, more subtle theories.

The idea of an association between stomach cancer and diet is not novel and has been suggested by many investigators. However, the relationship which may exist has not been simple or obvious enough to be detected through examination of gross differences in national diets, such as those undertaken by Herbert and Bruske (*84*) in England and the Netherlands and by Dungal (*21*, *85*) in Iceland. One difficulty may be that the data are not presented in detail for individual food items but have been grouped into such categories as meat, fish, milk products, vegetables, and fruits in order to consider specific hypotheses such as the effects of high and low protein diets. Also, the national data on diets have not been supplemented with evidence of variation among population subgroups or of differences between cancer patients and controls.

In principle, this is different from an approach that looks at individual food items, notes differences, and builds up leads for further testing from the detailed observations. In the search for diet effects, the first concern need not be for a specification of mechanisms producing the effect. Whatever the method of approach, it must be mediated through the consumption of certain foodstuff(s). This suggests that one should first look for differences in dietary histories of stomach-cancer patients and

of matched controls. It is a surprising fact that no serious efforts to collect dietary histories for cancer patients and matched controls have been made in the past; the work of Segi (*76*) and Quisenberry (*17*) is among the first steps in this direction. Among the more interesting results of Segi and his coworkers are their findings of a higher stomach-cancer risk among persons who use rice as a staple food compared to those who supplement rice with other cereals, and a lower risk among heavy users of two soybean products—shoyu and miso.

Data on variation in national diets may serve as a basis for selecting items to include in detailed studies of cancer patients and controls. The use of foodstuffs has been well documented in the United States by the Department of Agriculture (*86–88*), and table 14 summarizes some available facts on trends and variation (as determined from sample surveys) in per capita consumption by income, residence, and region. Since the table was viewed as a screening device, only differences on the order of 15 percent or more were considered noteworthy. The contrasts in the headings are so arranged that a "yes" entry indicates a difference in the same direction as that observed for stomach cancer and "no," a difference in the opposite direction. A review of individual food items and their nutrients does not single out any that correspond exactly, either directly or inversely, to the pattern of variation exhibited by stomach cancer. However, since much of the information relates to 1955, it may not always reflect the situation during an earlier period for currently diagnosed cases. Also, the per capita averages may conceal more concentrated use of food items among some population subgroups.

Some of the more striking features for the United States include the trend to greatly increased use of citrus fruits, tomato juice, and lettuce, the latter having displaced cabbage as the major green leafy vegetable; the decreasing consumption of potatoes and wheat flour; the larger amounts of beef, milk, citrus fruits, and green vegetables in high income diets; the higher consumption of potatoes in northern states, and of wheat flour, corn meal, and lard and other shortenings in southern states.

The results for the United States are supplemented in table 14 by data for 2 countries with high stomach-cancer rates—Japan and Finland. The diet in Iceland, another country with a high stomach-cancer rate, resembles that of Finland in many respects. While these data should be regarded as rough, qualitative indexes, in combination they may suggest some priorities for the inclusion of items in controlled retrospective studies of diets of stomach-cancer patients. The results do not suggest meat as a promising item of investigation despite past speculation on this topic. Even the data for smoked-pork products bear no consistent relation to the epidemiologic pattern for stomach cancer. Some other results favor this negative inference. Copeman and Greenwood (*90*) were unable to detect any departures from the general British experience in cancer mortality among members of religious orders with dietary rules restricting the use of meat. If the orders had had unusually low stomach- or digestive system-cancer rates, this effect ought to have been apparent

in the total cancer mortality. There is no evidence of unduly high rates for cancer of the digestive system among Canadian Indians (*37*) for whom meat forms a substantial part of the diet. The high stomach-cancer rates for northern countries is another indication for not anticipating an effect associated with meat, since climate would exert a greater influence on crops than on animal husbandry.

The last consideration would indicate that vegetables and fruits are more likely items for investigation. For example, the use of apples, which grow in colder climates, appears to be prevalent among several populations with high stomach-cancer rates. Citrus fruits, particularly oranges, have been displacing apples in the American diet during the period of declining stomach-cancer rates, and oranges are also more frequently used in high-income diets. This configuration may warrant further attention to apples and citrus fruits in retrospective studies. Lettuce does not grow well in cold climates and is absent from diets in Finland and Iceland where cabbage is a major green leafy vegetable. During the past 40 years, lettuce has displaced cabbage as the leading green leafy vegetable in the United States. Within the United States cabbage has been a staple in low-income diets, while lettuce is more characteristic of high-income diets. The major discrepancy in relating the use of lettuce and cabbage to the distribution of stomach cancer is the greater use of cabbage in the southern states.

These comments involve selection and interpretation of the recorded facts which all observers would not necessarily subscribe to and must be regarded as in the realm of speculation. They are mentioned primarily as illustrations of how an investigator might acquire ideas which can be probed further by studies of diagnosed cases and matched controls.

The collection of dietary histories poses formidable difficulties. Because of uncertainty as to the nature of the effect being sought, they must in the initial phases, at least, deal with individual food items and their preparation. There is the added complication that certain items in the diet imply the absence of others. Because of the probably long latent period between exposure and development of disease, the dietary histories ought to cover long periods, which would, however, subject them to error. Some family members may have inadequate knowledge and would not be reliable respondents. In certain areas some items of diet will be in such widespread use that it might be impossible to demonstrate any differences between cancer patients and controls, even if a relationship existed. On the other hand, some items of diet may be associated with many population characteristics, and differences between cancer patients and controls might be noted which were attributable to indirect associations with other population characteristics and devoid of etiologic significance. Under these circumstances the results from any single study may prove misleading.

Some of these difficulties could be mitigated by refining study techniques. Inaccuracies in dietary histories might be reduced by interviewing more than one family member. The quality of responses might be

TABLE 14.—*Variation in per capita consumption of foodstuffs and nutrients in the United States, supplemented by observations for Japan and Finland**

Foodstuffs and nutrients	Per capita consumption of item in United States					Item commonly or frequently used in diets of:	
	Downward trend 1909–54 † (*86*)	Higher in low income families, 1955 (*87*)	Higher in farm families, 1955 (*87*)	Higher in northern states		Japan	Finland
				1955 (*87*)	1936 (*88*)		
Beef	—	No	—	Yes	Yes	Yes	Yes
Veal	—	No	No	Yes		No	No
Lamb	Yes	No	No	Yes		No	No
Pork	—	—	Yes	—	No	Yes	Yes
Ham		No	—	—		No	Yes
Bacon		No	Yes	—		No	No
Salt pork		Yes	Yes	No		No	No
Chicken	No	—	—	No	No	Yes	No
Turkey	No	No	No	Yes		No	No
Eggs	No	—	Yes	—	Yes	Yes	No
Fish, fresh	—	Yes	—	No		Yes	Yes
Shellfish	—	No	No	No		Yes	Yes
Salmon	—	—	—	—			No
Tuna	No	No	No	—			No
Fish, salted						Yes	Yes
Milk, fluid equivalent	—	No	Yes	—	Yes	No	Yes
Cheese	No	No	No	Yes		No	Yes
Ice cream	No	No	—	Yes		No	No
Butter	Yes‡	No	Yes	Yes	—	No	Yes
Margarine	No	—	No	—		No	Yes
Lard	—	Yes	Yes	No	No	No	No
Other shortening	—	—	—	No		Yes	No
Fruits (fresh, canned, frozen, dried)							
Citrus fruits	No	No	No	Yes			
Grapefruit		No	No	Yes		No	No
Lemons, limes		—	—	No		Yes	No
Oranges		No	No	Yes		Yes	Yes

Apples	Yes	—	—	Yes		Yes	Yes
Bananas		No	—	—		No	No
Watermelon	Yes	—	No	No		No	No
Cantaloupe	—					No	No
Rhubarb		—	Yes	Yes		No	No
Strawberries		No	Yes	Yes		No	Yes
Cherries		—	Yes	Yes		No	No
Peaches		—	Yes	Yes		No	No
Pineapple		No	—	Yes		No	No
Prunes		—	—	—		No	Yes
Raisins		—	Yes	—		No	Yes
Vegetables (fresh, canned, frozen, dried)							
Tomatoes, tomato juice	No	No	—	Yes		No	Yes
Catsup, chili sauce, paste	No	No	—	Yes			
Asparagus	—	No	No	Yes			No
Beans, lima	No	—	—	No			No
Beans, lima (dried)		Yes	Yes	No			
Beans, snap	No	Yes	Yes	No			No
Beans, navy, kidney		Yes	Yes	No		Yes	No
Broccoli	No	No	No	Yes		No	No
Cabbage	Yes	Yes	Yes	No		Yes	Yes
Carrots	No	No	No	Yes		Yes	Yes
Lettuce, escarole	No	No	—	Yes		Yes	No
Peas	No	—	Yes	No		No	Yes
Peas (dried)		Yes	—	No			
Peppers	No	No	No	—		No	No
Spinach	—	No	—	—		No	No
Beets	—	No	No	No		Yes	Yes
Celery	No	No	No	Yes		No	No
Corn	No	—	—	No		No	No
Cucumbers	—	No	No	Yes		Yes	Yes
Pickles	No	No	Yes	Yes			
Onions	—	—	—	—		Yes	Yes
Potatoes	Yes	—	Yes	Yes	Yes	Yes	Yes
Sweet potatoes	Yes§	Yes	—	No		Yes	No
Olives		No	No	Yes		No	No

See footnotes at end of table.

TABLE 14.—*Variation in per capita consumption of foodstuffs and nutrients in the United States, supplemented by observations for Japan and Finland**—Continued

Foodstuffs and nutrients	Per capita consumption of item in United States					Item commonly or frequently used in diets of:	
	Downward trend 1909–54 † (*86*)	Higher in low income families, 1955 (*87*)	Higher in farm families, 1955 (*87*)	Higher in northern states		Japan	Finland
				1955 (*87*)	1936 (*88*)		
Wheat flour	Yes	Yes	Yes	No	No	Yes	Yes
Rice	Yes	Yes	Yes	No		Yes	Yes
Cornmeal	Yes	Yes	Yes	No	No	No	No
Oat products	—	Yes	Yes	—		No	Yes
Rye flour						No	Yes
Sugar	—	Yes	Yes	—	—	Yes	Yes
Corn syrup	—	Yes	Yes	No		No	No
Molasses	Yes	Yes	Yes	No		No	No
Coffee	No	No	—	Yes		No	Yes
Tea	Yes	—	—	No		Yes	No
Cocoa beans	No	No	—	—		No	No
Peanuts	No	No	—	Yes		Yes	No
Soft drinks, cola flavored		No	—	No			
Soft drinks, fruit flavored		No	No	Yes			
Nutrients							
Food energy	—	—	Yes		No		
Protein	—	No	—		—		
Fat	No	No	Yes				
Calcium	No	—	Yes		No		
Iron	—	—	—				
Vitamin A	No	No	No		No		
Thiamin	No‡	—	Yes		—		

Riboflavin	No‡	—	Yes		—		
Niacin	—	—	—				
Folic acid	—						
Ascorbic acid	No‖	No	No		Yes		

*Table summarizes findings for the United States from sources cited. Where necessary, amounts per household were converted into amounts per capita, using the published data on size of household. Miss Jean McNaughton, of the Food and Agriculture Organization, and Miss Eeva Korhonen, of the Public Health Service of Finland, kindly supplied information on diets in Japan and Finland. Reference was also made to Segi's summary (*76*) of a 1954 nutrition survey in Japan and Rautavaara's review (*89*) of the Finnish diet. Within the United States differences of small magnitude, generally less than 10–15 percent, have been ignored. The entries should be interpreted as follows:

Yes = Difference in the direction indicated by column heading;
No = Difference in opposite direction from that indicated by column heading;
— = No major change or difference;
Blank = No data.

†Trends for most vegetables based on data for 1919–54.
‡Most of the change occurred after 1942.
‖Most of the change occurred after 1946.
¶The upward trend was reversed after 1945.

improved by dealing with a limited range of food items in any single spectrum. Another interesting approach would be to collect diet histories from population groups with a known excess of stomach-cancer risk such as persons with achlorhydria. If achlorhydria may be regarded as a precursor, more reliable histories could be collected for the years preceding the initiation of the disease. Comparison of controlled studies of achlorhydric patients with those of gastric-cancer patients might provide further insight into the effects of diet. Such refinements as whether there were differences between achlorhydric patients who did and who did not subsequently develop stomach cancer could also be attempted. The development of tubeless gastric analysis as a screening test for achlorhydria makes possible these latter variations.

The basic protection against erroneous inferences, however, must come through cross-checking the results of several controlled retrospective studies of stomach-cancer patients which would permit assessment of the influence of the uncontrolled variables in different study settings and, by collation of data, narrow successively the field of inquiry.

These considerations require that information on many items should be common to several studies, though the need for replication and overlapping of results does not mean that complete uniformity in all respects is necessary or desirable. The requirements can best be met by coordination and exchange of information among several investigators studying the relationship of diet and other factors with stomach cancer. Specifically, if an investigator found an item that discriminated strongly between diagnosed cases and controls, he might wish some of his confreres to try it while his work was still in progress. Because of the long time required to initiate and collect field and clinical data on human populations as compared to that needed for animal experimentation, the traditional dependence on published papers for the exchange of definitive data could mean substantial delays in arriving at the facts. Faster progress might be made if an organization such as the National Cancer Institute took the initiative in the formation and support of a group of investigators for the purpose of coordinating and exchanging information from epidemiologic studies of stomach cancer, with suitable arrangements for recognizing priority in publications.

Despite the difficulties, a series of controlled retrospective studies of the diet of stomach-cancer patients offers possibilities which should be exploited in the absence of more promising alternatives. The collection of data in controlled studies may resolve some apparent inconsistencies in the crude information now available, and lead to the isolation of some food item or combination which would fit into the total epidemiologic pattern for stomach cancer. The findings might then be tested in other ways, such as observations on defined cohorts exposed to specific conditions or possibly the development of experimental biological response situations.

References

(1) Doll, R.: Environmental factors in the aetiology of cancer of the stomach. Gastroenterologia 86: 320–328, 1956.

(2) Haenszel, W., Marcus, S. C., and Zimmerer, E. G.: Cancer Morbidity in Urban and Rural Iowa. Pub. Health Monogr. No. 37, Pub. Health Ser. Publ. No. 426. Washington, D. C., U. S. Gov't. Print. Office, 1956, pp. 85.

(3) Dorn, H. F., and Horn, J. I.: The reliability of certificates of deaths from cancer. Am. J. Hyg., Sect. A, 34: 12–23, 1941.

(4) Hoffman, F. L.: The mortality from cancer throughout the world. Newark, Prudential Press, 1915.

(5) Segi, M.: Observation on the mortality from ulcer of stomach and duodenum on the basis of the vital statistics data. Department of Public Health, Tohoku University School of Medicine, Sendai, Japan, 1957. (A part of the report presented to the International Society of Geographical Pathology by Dr. T. Kurokawa and Dr. A. Okabayashi.)

(6) Neurdenberg, M. G.: On carcinoma of the stomach. Schweiz. Ztschr. allg. Path. 18: 507–537, 1955.

(7) Dunham, L. J., and Dorn, H. F.: Techniques in the geographic pathology of cancer. Schweiz. Ztschr. allg. Path. 18: 472–481, 1955.

(8) Clemmesen, J., and Nielsen, A.: The incidence of malignant diseases in Denmark 1943 to 1947. Acta Unio internat. contra cancrum, special No., 8: 140–159, 1952.

(9) Saxén, E.: Report from the Finnish cancer registry. Schweiz. Ztschr. allg. Path. 18: 556–562, 1955.

(10) Eastcott, D. F.: Report of the B. E. C. C. (N. Z.) Branch, Cancer Registration Scheme. Department of Health, Wellington, New Zealand, 1954.

(11) Breslow, L.: Epidemiology as a tool in cancer research. *In* Proceedings of the Third Nat. Cancer Conference, Detroit, Mich. Philadelphia, J. B. Lippincott Co., 1956, pp. 15–26.

(12) Griswold, M. H., Wilder, C. S., Cutler, S. J., and Pollack, E. S.: Cancer in Connecticut, 1935–1951. Hartford, Connecticut State Dept. Health, 1956, 41 pp.

(13) Levin, M. L., Haenszel, W., Carroll, B. E., Gerhardt, P. R., Handy, V. H., and Ingraham, S. C.: Cancer incidence in urban and rural areas of New York State. To be published.

(14) Segi, M., Fukushima, I., Fujisaku, S., Kurihara, M., Saito, S., Asano, K., and Nagaike, H.: Cancer morbidity in Miyagi Prefecture, Japan, and a comparison with morbidity in the United States. J. Nat. Cancer Inst. 18: 373–383, 1957.

(15) Dorn, H. F., and Cutler, S. J.: Morbidity from Cancer in the United States. Pub. Health Monogr. No. 29. Washington, D. C., U. S. Gov't. Print. Office, 1956, 121 pp.

(16) Smith, R. L.: Recorded and expected mortality among Japanese of the United States and Hawaii, with special reference to cancer. J. Nat. Cancer Inst. 17: 459–473, 1956.

(17) Quisenberry, W. B.: Stomach cancer in Hawaii. M. Arts & Sc. 9: 74–81, 1955.

(18) Steiner, P. E.: Cancer: Race and Geography. Baltimore, Williams and Wilkins, 1954, 363 pp.

(19) Phillips, A. J., and Owchar, M.: Mortality trends in Canada for various sites of cancer. Canad. M. A. J. 73: 626–630, 1955.

(20) Rakower, J.: Lung cancer in Israel. Cancer 10: 67–71, 1957.

(21) Dungal, N.: Cancer in Iceland, with special reference to stomach cancer. Schweiz. Ztschr. allg. Path. 18: 550–556, 1955.

(22) Correa, P.: Statistical study of cancer in Antioquia. Schweiz. Ztschr. allg. Path. 18: 491–500, 1955.

(23) U. S. Public Health Service, National Office of Vital Statistics: Vital Statistics of the United States. Annual reports for the years 1949–1951. Washington, D. C., U. S. Gov't. Print. Office.

(24) Dominion Bureau of Statistics. Vital Statistics Annual Report, 1951. Ottawa, Ontario, Canada.

(25) SEGI, M.: Cancer Mortality Statistics in Japan. Dept. Public Health, Tohoku University School of Medicine, Sendai, Japan, 1955.

(26) ARMITAGE, P., and DOLL, R.: The age distribution of cancer and multistage theory of carcinogenesis. Brit. J. Cancer 8: 1–12, 1954.

(27) BARRETT, M. K.: Avenues of approach to the gastric-cancer problem. J. Nat. Cancer Inst. 7: 127–157, 1946.

(28) TROMP, S. W.: The geographical distribution of cancer of the stomach in the Netherlands (Period 1946–1952). Brit. J. Cancer 10: 265–281, 1956.

(29) STOCKS, P.: A study of the age curve for cancer of the stomach in connection with a theory of the cancer producing mechanism. Brit. J. Cancer 7: 407–417, 1953.

(30) GERHARDT, P. R., HANDY, V. H., and FERBER, B.: Trends in cancer incidence, mortality and probability in the State of New York. New York J. Med. 57: 1387–1390, 1957.

(31) Unpublished data from 10-city cancer morbidity surveys.

(32) CASE, R. A. M.: Cohort analysis of cancer mortality in England and Wales, 1911–1954, by site and sex. Brit. J. Prev. & Social Med. 10: 172–199, 1956.

(33) SMITH, R. L.: Recorded and expected mortality among the Chinese of Hawaii and the United States with special reference to cancer. J. Nat. Cancer Inst. 17: 667–676, 1956.

(34) ———: Recorded and expected mortality among the Indians of the United States with special reference to cancer. J. Nat. Cancer Inst. 18: 385–396, 1957.

(35) ———: Mortality attributed to cancer among Hawaiians and Filipinos of Hawaii and other racial groups of the United States and Hawaii. J. Nat. Cancer Inst. 18: 397–405, 1957.

(36) GORDON, T.: Mortality experience among the Japanese in the United States, Hawaii, and Japan. Pub. Health Rep. 72: 543–553, 1957.

(37) WARWICK, O. H., and PHILLIPS, A. J.: Cancer among Canadian Indians. Brit. J. Cancer 8: 223–230, 1954.

(38) LOMBARD, H. L., and DOERING, C. R.: Cancer studies in Massachusetts. III. Cancer mortality in nativity groups. J. Prev. Med. 3: 343–361, 1929.

(39) STOCKS, P.: Regional and local differences in cancer death rates. Studies on Medical and Population Subjects, No. 1. London, Her Majesty's Stat. Off., 1947.

(40) SPIEGELMAN, M.: Demographic differentials in cancer mortality in urban and rural areas of the United States, 1940. National Office Vital Statistics, Special Reports 33: 239–254, 1952.

(41) CLEMMESEN, J., and NIELSEN, A.: Comparison of age-adjusted cancer incidence in Denmark and the United States. J. Nat. Cancer Inst. 19: 989–998, 1957.

(42) OLINDER, O.: Kanserdodeligheten i stadene och pa landsbygden. Statistisk Manadsskrift for Stockholm stad nr. 10, 1953.

(43) STOCKS, P.: Cancer of the stomach in the large towns of England and Wales, 1921–1939. Brit. J. Cancer 4: 147–157, 1950.

(44) ———: Cancer and goitre. Biometrika 16: 364–401, 1924.

(45) TROMP, S. W., and DIEHL, J. C.: A statistical study of the possible relationship between cancer of the stomach and soil. Brit. J. Cancer 9: 349–357, 1955.

(46) LEGON, C. D.: A note on geographical variations in cancer mortality, with special reference to gastric cancer in Wales. Brit. J. Cancer 5: 175–179, 1951.

(47) DAVIES, R. I., and WYNNE GRIFFITH, G.: Cancer and soils in the county of Anglesey. Brit. J. Cancer 8: 56–66, 1954.

(48) KRAUS, A. S., LEVIN, M. L., and GERHARDT, P. R.: A study of occupational associations with gastric cancer. Am. J. Pub. Health 47: 961–970, 1957.

(49) PATNO, M. E.: Geographic study of cancer prevalence within an urban population. Pub. Health Rep. 69: 705–715, 1954.

(50) DORN, H. F., and CUTLER, S. J.: Morbidity from Cancer in the United States, Part II. Pub. Health Monogr. Washington, D. C., U. S. Gov't. Print. Office. In press.

(51) COHART, E. M.: Socioeconomic distribution of stomach cancer in New Haven. Cancer 7: 455–461, 1954.

(52) CLEMMESEN, J., and NIELSEN, A.: The social distribution of cancer in Copenhagen, 1943 to 1947. Brit. J. Cancer 5: 159–171, 1951.

(53) RENNAES, S., and ØSTBERG, E. W.: Cancer ventriculi mortality in different population groups. Cancer ventriculi mortality in various parts of Oslo, 1930–1950. Brit. J. Cancer 9: 7–20, 1955.

(54) TORGERSEN, O., and PETERSEN, M.: The epidemiology of gastric cancer in Oslo: material, diagnosis, and mobility of population. Some sources of error. Brit. J. Cancer 10: 292–298, 1956.

(55) (———): The epidemiology of gastric cancer in Oslo: cartographic analysis of census tracts and mortality rates of sub-standard housing areas. Brit. J. Cancer 10: 299–306, 1956.

(56) LOGAN, W. P. D.: Social class variations in mortality. Pub. Health Rep. 69: 1217–1223, 1954.

(57) YOUNG, M., and RUSSELL, W. T.: An investigation into the statistics of cancer in the different trades and professions. M. Res. Council Spec. Rep. No. 99. London, His Majesty's Stat. Off., 1926.

(58) VIDEBAEK, A., and MOSBECH, J.: The aetiology of gastric carcinoma elucidated by a study of 302 pedigrees. Acta med. scandinav. 149: 137–159, 1954.

(59) MACKLIN, M. T.: The role of heredity in gastric and intestinal cancer. Gastroenterology 29: 507–514, 1955.

(60) (———): Inheritance of cancer in man. Schweiz. Ztschr. allg. Path. 18: 463–471, 1955.

(61) HANHART, E.: Auffallend geringe Bedeutung der "Belastung mit Krebs." Schweiz. med. Wchnschr. 7: 446–449, 1943.

(62) WOOLF, C. M.: Investigations on genetic aspects of carcinoma of the stomach and breast. Univ. California Pub. Health 2: 265–350, 1955.

(63) STATE, D., VARCO, R. L., and WANGENSTEEN, O. H.: An attempt to identify likely precursors of gastric cancer. J. Nat. Cancer Inst. 7: 379–384, 1947.

(64) HAGY, G. W.: A familial study of gastric carcinoma. Am. J. Human Genet. 6: 434–447, 1954.

(65) GREENWOOD, M., and YULE, G. U.: On the determination of size of family and of the distribution of characters in order of birth from samples taken through members of the sibships. Roy. Stat. Soc. J. 77: 179–197, 1914.

(66) DUBLIN, L. I., and MARKS, H. H.: The inheritance of longevity—A study based upon life insurance records. Proc. Assoc. Life Insurance Medical Directors of America 28: 89–120, 1941.

(67) MIDER, G. B.: An introduction to cancer research. Am. J. Med. 8: 71–89, 1950.

(68) HITCHCOCK, C. R., SULLIVAN, W. A., and WANGENSTEEN, O. H.: The value of achlorhydria as a screening test for gastric cancer. A 10-year report. Gastroenterology 29: 621–632, 1955.

(69) BERKSON, J., COMFORT, M. W., and BUTT, H. R.: Occurrence of gastric cancer in persons with achlorhydria and with pernicious anemia. Mayo Clin., Proc. Staff Meet. 31: 583–596, 1956.

(70) DOEHRING, P. C.: Carcinoma of the stomach associated with pernicious anemia. West. J. Surg. 62: 391–394, 1954.

(71) AIRD, I., BENTALL, H. H., and ROBERTS, J. A. F.: A relationship between cancer of stomach and the ABO blood groups. Brit. M. J. 1: 799–801, 1953.

(72) BILLINGTON, B. P.: Gastric cancer—relationships between ABO blood-groups, site, and epidemiology. Lancet 2: 859–862, 1956.

(73) BUCKWALTER, J. A., WOHLWEND, C. B., COLTER, D. C., TIDRICK, R. T., and KNOWLER, L. A.: The association of the ABO blood groups to gastric carcinoma. Surg., Gynec. & Obst. 104: 176–179, 1957.

(74) KOSTER, K. H., SINDRUP, E., and SEELE, V.: ABO blood-groups and gastric acidity. Lancet 2: 52–55, 1955.

(75) SPEISER, P.: Bestehen mathematisch gesicherte Beziehungen der A-B-O Gruppen, des Rhesusfaktors Rh_o (D) und des Geschlechtes zu Carcinoma ventriculi, Ulcus ventriculi und Ulcus duodeni? Krebsarzt 11: 344–348, 1956.

(76) SEGI, M., FUKUSHIMA, I., FUJISAKU, S., KURIHARA, M., SAITO, S., ASANO, K., and KAMOI, M.: An epidemiological study on cancer in Japan. Gann 48: Supp., 1957.

(77) AIRD, I., BENTALL, H. H., MEHIGAN, J. A., and ROBERTS, J. A. F.: The blood groups in relation to peptic ulceration and carcinoma of colon, rectum, breast, and bronchus; an association between the ABO groups and peptic ulceration. Brit. M. J. 2: 315–321, 1954.

(78) CORNFIELD, J.: A method of estimating comparative rates from clinical data. Applications to cancer of the lung, breast, and cervix. J. Nat. Cancer Inst. 11: 1269–1275, 1951.

(79) HAMMOND, E. C., and HORN, D.: The relationship between human smoking habits and death rates. J. A. M. A. 155: 1316–1328, 1954.

(80) HAENSZEL, W., SHIMKIN, M. B., and MILLER, H. P.: Tobacco Smoking Patterns in the United States. Pub. Health Monogr. No. 45., Pub. Health Ser. Publ. No. 463. Washington, D. C., U. S. Gov't. Print. Office., 1956, pp. 111.

(81) Registrar-General of England and Wales: The Registrar-General's Decennial Supp., England and Wales 1931. IIA. Occupational Mortality. London, 1938.

(82) U. S. Dept. Health, Education, and Welfare, National Office Vital Stat.: Mortality from selected causes by marital status. United States, 1949–1951. Vital Statistics Special Report 39: 303–429, 1956.

(83) BOYD, J. T., and DOLL, R.: Gastro-intestinal cancer and the use of liquid paraffin. Brit. J. Cancer 8: 231–237, 1954.

(84) HERBERT, W. E., and BRUSKE, J. S.: Etiology of cancer of the stomach. II. A comparison of the diet and dental condition of the English and Dutch, with special reference to gastric irritants. Guy's Hosp. Rep. 86: 301–308, 1936.

(85) DUNGAL, N.: Studies on diet in relation to cancer. British Empire Cancer Campaign, 24th Ann. Rep.: 119–120, 1956.

(86) U. S. Dept. Agriculture. Agriculture Handbook No. 62. Supp. for 1954 to Consumption of Food in the United States, 1909–1952.

(87) *Ibid*: Household Food Consumption Survey 1955.
Report No. 1: Food consumption of households in the United States.
Report No. 2: Food consumption of households in the Northeast.
Report No. 3: Food consumption of households in the North Central Region.
Report No. 4: Food consumption of households in the South.
Report No. 5: Food consumption of households in the West.
Report No. 6: Dietary levels of households in the United States.

(88) *Ibid*: Consumer Purchases Study, 1936. Family food consumption and dietary levels. Farm Series, Misc. Publ. No. 405; Urban and Village Series, Misc. Publ. No. 452.

(89) RAUTAVAARA, T.: Food in Other Lands—Finland. Food and Nutrition 5: 12–14, 1950.

(90) COPEMAN, S. M., and GREENWOOD, M.: Diet and cancer, with special reference to the incidence of cancer upon members of certain religious orders. Report on Public Health and Medical Subjects No. 36. London, His Majesty's Stat. Off., 1926.

Effect of Actinomycin D on Tissue Cultures of Normal and Neoplastic Cells [1,2,3]

JEWEL PLUMMER COBB *and* DOROTHY G. WALKER,
Department of Surgery, New York University Post-Graduate Medical School, the Fourth Surgical Division (N. Y. U.) of Bellevue Hospital and the University Hospital, New York, New York

Summary

J. Nat. Cancer Inst. 21: 263–277, 1958

Actinomycin D produced a range of nonspecific cytotoxic changes in human neoplastic and normal primary tissue cultures.

Neoplastic primary cultures were generally more sensitive to the action of actinomycin D than normal primary cultures, as judged by their cytological changes. On the other hand, both showed marked reduction in mitosis.

Both neoplastic and normal established cell strains showed only mild sensitivity to the same concentrations of actinomycin D that damaged primary cultures. Sensitivity was indicated by an increase in the number of aberrant metaphases. There were relatively fewer aberrant metaphases in treated HeLa cultures as compared with conjunctiva and kidney cultures.

A selective response to actinomycin D has been shown to exist among the primary cultures included in this study. In view of these observations further study of the use of primary cultures for evaluating the ***in vitro*** effects of a potential chemotherapeutic agent is suggested.

This investigation describes the biological activity of the antibiotic actinomycin D in a variety of cellular test systems. These include: (*a*) primary short-term tissue cultures of mouse embryo heart, and human normal and neoplastic tissues, and (*b*) established cell lines of human normal and neoplastic origin. These culture systems were chosen with the aim of revealing additional information regarding the biological activity of the drug.

The antibiotic actinomycin D is one of a group of closely related actinomycins. Actinomycin was first isolated by Waksman and Woodruff in 1940 (*1*) from a culture of soil actinomyces. Since that time a number

[1] Received for publication July 8, 1957.

[2] Presented in part at the 48th annual meeting of the American Association for Cancer Research at Chicago, Ill., April, 1957.

[3] This investigation was supported by research grant C-2780 from the National Cancer Institute of the National Institutes of Health, Public Health Service, and in part by the Damon Runyon Memorial Fund for Cancer Research, Inc.

of actinomycins have been recognized. All are similar in the possession of the same red chromophore, 4,7-dihydroxy-3,6-dimethylacridone-5, 8-quinone, which was elucidated by Brockmann and Muxfeldt (*2*, *3*) in 1955. They differ from one another in the composition of the peptide side chain linked to the chromophore. Actinomycin D was isolated and described by Manaker, Gregory, Vining, and Waksman in 1954 (*4*).

Early work utilizing actinomycin C against a number of rat and mouse neoplasms gave encouraging results (*5–7*). These results led to their further use in clinical trials (*8*, *9*).

Farber in 1955 (*10*) first reported the antitumor activity of actinomycin D on myeloid and lymphoid leukemia in mice. A marked effect of the drug on experimental ascites tumors in mice was observed by Gregory *et al.* in 1956 (*11*). Pronounced tumor inhibition of a mouse glioblastoma, the S91 melanoma, and the 6C3HED Gardner lymphosarcoma was observed, following actinomycin D administration, by Fantini and colleagues (*12*).

Clinical studies by Farber, Maddock, and Swaffield have shown important temporary changes in children with rhabdomyosarcoma, Wilms' tumor, and Hodgkin's disease (*13*). Golomb, Gumport, and Wright, in treating 61 adults, noted significant objective improvement in one case each of malignant melanoma, Hodgkin's disease, lymphosarcoma, and chronic lymphatic leukemia, and, in children, 1 case of Wilms' tumor (*14*).

Eagle and Foley demonstrated the cytotoxic activity of actinomycin D *in vitro* against 6 established cell lines derived from human or mouse normal and neoplastic tissue. There was no apparent correlation between the cytotoxicity in tissue culture and either acute toxicity in mice or antibacterial activity in the specific assay systems used (*15*).

Materials and Methods

Mouse heart tissue was obtained from *in utero* strain DBA or BALB/cAnN mice. One mm.^{3} fragments were embedded in a soft coagulum consisting of fowl plasma and chick-embryo extract on double coverslips on Maximow slides. One drop of nutrient medium, composed of 90 percent Eagle's basal medium (EBM) supplemented with 10 percent nondialyzed horse or human serum, was added to each coverslip. Twice each week, the old fluid medium was withdrawn and fresh medium added.

Human neoplastic tissue was excised by sterile procedures from patients with disseminated or advanced neoplastic disease. Normal lymph nodes were obtained from patients undergoing surgery for either a herniorraphy or varicose-vein ligation and stripping. Both tissue types were cut in 1 to 2 mm.^{3} fragments and embedded in a plasma-embryo extract coagulum on 12 $\times$ 20 mm. coverslips, which were inserted in Porter flasks. One ml. of nutrient medium, EBM supplemented with 10 percent nondialyzed serum, was added to each flask. Pleural or ascitic-effusion specimens from patients with advanced neoplastic disease were centrifuged at 2000 r.p.m. for 10 minutes. The supernate was withdrawn, and the sedimented cells dispensed in 0.2 ml. aliquots to several Gey roller-tubes or Porter flasks

without plasma. One ml. of nutrient medium, consisting of 50 percent EBM plus horse serum plus 50 percent clear, autologous pleural or ascitic fluid, was added. The medium for all cultures of human origin was renewed twice each week and the *p*H adjusted to 7.2 before use. In this group only actively growing primary cultures (1 to 36 days *in vitro*) were employed for both control and drug-study series.

The rate and extent of growth was recorded for each tissue series before drug treatment. The growth rate is defined as the average distance cells emigrated from the explant and/or the average density of the same cell population, per 72-hour-interval examinations. The extent of outgrowth is defined as the total distance cells emigrated at time of drug treatment irrespective of unit time. Qualitative linear measurements (taken from the average of 2 parameters per explant) of the axes of outgrowth distances were made at 100× magnification with an ocular micrometer. The units were expressed in microns. The cell-population density was roughly determined by inserting a Howard grid in the eyepiece and estimating the number of cells in two 150× fields of each outgrowth.

The established cell strains used here were obtained from two sources. Gey's neoplastic epithelial-cell line HeLa was obtained in test tubes from Carver Research Foundation, Tuskegee, Alabama, where it has been maintained in continuous culture for commercial distribution. The human conjunctiva and kidney tissue cells originally cultured by Chang are now established cell lines. These lines were obtained as bottle cultures from the South Jersey Medical Research Center where they have been serially propagated.[4] In our laboratory they were cultivated without plasma on one side of Porter flasks containing 2 oblong coverslips. The medium, composed of 90 percent EBM and 10 percent horse serum, was apportioned in 1 ml. aliquots per flask and renewed twice weekly. New flasks were seeded with cells by the following method: The old nutrient medium was removed from the cells in tubes and 1 ml. of 0.125 percent trypsin (Difco 1:250) added. The *p*H was 7.4. The cultures containing trypsin were incubated 10 minutes at 37° C. After gentle agitation, 0.005 percent soybean-trypsin-inhibitor was added and tubes were centrifuged at 2660 r.p.m. for 10 minutes. The supernate was withdrawn, and the cells were suspended in fresh medium and counted. A dilution of approximately 100,000 cells was planted in each flask. Only those flasks bearing coverslips covered with an even, continuous cell layer were used.

Throughout this study only primary or established cell-line cultures with a minimum cell population of 1,000 cells were selected for drug experiments. Each tissue type always had an equivalent control group. Actinomycin D [5] was prepared from a powder and 20 mg. was dissolved in 2 ml. of absolute alcohol and 198 ml. of isotonic saline solution added to make a concentration of 100 μg. per ml. This stock solution was

[4] The authors wish to thank Dr. Lewis L. Coriell, of the South Jersey Medical Research Foundation, Camden, N. J., for generously supplying conjunctiva and kidney cell cultures.

[5] Actinomycin D used in this investigation was furnished through the courtesy of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

sterilized by filtration through a Corning fritted-glass filter of UV porosity. After several serial dilutions in nutrient medium, the drug was added to cultures as a 1 to 9 dilution. Mouse heart cultures were exposed to various concentrations under a regime to be described. Human cells from normal and neoplastic primary tissue and established cell lines were exposed to 1×10^{-3} and 1×10^{-4} μg. per ml. concentrations of the drug for 48 or 96 hours when they were either washed and refed for recovery studies or fixed. Fixed preparations were either stained with Jenner-Giemsa or subjected to the Feulgen reaction.

Results

Preliminary Studies

Mouse embryo heart and HeLa cultures were used in the initial screening of actinomycin D to establish an effective range of concentrations for further experiments. Concentrations tested were from 1×10^{-6} to 10 μg. per ml. Results of drug studies with mouse heart cultures are summarized as follows: 1) Migration and mitosis were irreversibly checked in initial cultures treated for 96 hours with concentrations ranging from 1×10^{-4} to 10 μg. per ml. Pulsation was not checked, however, during or after drug exposure. 2) Actinomycin D, at 1×10^{-4} to 1×10^{-6} μg. per ml. concentrations, produced marked cytotoxic changes in 5-day-old cultures after exposure for 48 hours. 3) Minced heart fragments that were suspended for 48 hours in nutrient medium with the drug concentration ranging from 1×10^{-4} to 1×10^{-2} μg. per ml. and then washed and embedded in a plasma clot on coverslips failed to grow after drug removal, while control fragments developed luxuriant outgrowths. 4) Inactivation studies which utilized a known toxic drug concentration indicated that actinomycin D was stable and active for 6 days at 37° C. in nutrient medium.

Only slight cytotoxic changes were produced, however, in HeLa cultures exposed to actinomycin D at concentrations that ranged from 1×10^{-5} to 1×10^{-3} μg. per ml. Detailed accounts of these changes will be reported in subsequent paragraphs. Therefore, based on the above preliminary findings with a human cell type, a concentration of 1×10^{-4} μg. per ml. was chosen as the dose expected to reveal subtle changes in other human-cell systems under investigation. This concentration is equivalent to 8.3×10^{-8} mmoles based on recent molecular-weight calculations of Dr. Modest.[6]

Cellular Changes in Primary Cultures

The first phase of this report deals with cellular changes in primary human cultures exposed to low concentrations of actinomycin D, namely, 1×10^{-4} and 1×10^{-3} μg. per ml. These changes were evaluated by the following criteria: granulation, vacuolation, pyknosis of resting or dividing

[6] Personal communication from Dr. George E. Foley, Children's Cancer Research Foundation, Boston, Mass.

cells, cytolysis, increased cellular debris, loss of staining capacity, nucleolar changes, mitotic-index changes, and drug reversibility.

Table 1 summarizes the cellular changes observed in 19 primary neoplastic tissue cultures exposed to the drug for 96 hours. The extent of cellular damage is indicated in 3 columns. Sensitivity of cells to the drug, or cytotoxicity, is recorded in column 3 as 1 plus (+) to 4 plus (++++), indicating the degree of sensitivity, or in column 5 as a plus-minus (±), indicating questionable sensitivity, or in column 4 as a minus (−), when cells were resistant.

TABLE 1.—*Summary of cellular response of primary human neoplastic cells exposed to actinomycin D*

Tissue type	Number	Number sensitive (+− ++++)	Number resistant (−)	Equivocal (±)
Fibrosarcoma	2	1		1
Liposarcoma	1	1		
Kaposi's sarcoma	1			1
Melanoma	4	2		2
Carcinoma of mouth	2		2	
Carcinoma of esophagus	1		1	
Carcinoma of breast	1	1		
Carcinoma of ovary	1		1	
Carcinoma of kidney	1	1		
Carcinoma (primary site unknown)	1		1	
Hodgkin's disease	1		1	
Myelogenous leukemia	1	1		
Chronic lymphatic leukemia	2	1		1
Total	19	8	6	5

Only 2 of 7 carcinoma series showed cytotoxic changes. These changes consisted of decreased nucleolar size, loss of staining capacity, and/or decreased mitoses.

Cytotoxicity was observed in 2 of 4 lymphoma series. Cells of a chronic lymphatic leukemia lymph node displayed decreased nucleolar size among other changes. Nucleolar changes in treated cells are shown in figure 2. The untreated cells are seen in figure 1. Slight changes occurred in treated splenic tissue containing myelogenous leukemic cells. As seen in table 1, actinomycin D elicited a range of cytotoxic changes apparently related to the individual patient source rather than the pathological tissue type.

Only mild cytotoxic changes, consisting of mitotic or nucleolar alterations, were observed in 4 of the 5 human normal tissue-culture series.

TABLE 2.—*Comparison of tissue-culture growth and drug response*

Tissue type	Number	Rate of growth*	Growth extent†	Cytotoxicity
Liposarcoma	52	Slow	+	S ++++
Fibrosarcoma	67	Moderate	++	S +
Fibrosarcoma	64B	Fast	++++	±
Kaposi's sarcoma	109	Moderate	+++	±
Melanoma	43	Slow	+	S ++++
Melanoma	110	Moderate	++	R −
Melanoma	122	Moderate	+++	R −
Melanoma	102	Fast	++++	S ++++
Carcinoma of mouth	112	Fast	++	R −
Carcinoma of tongue	119	Moderate	++	R −
Carcinoma of esophagus	46B	Fast	+++	R −
Carcinoma of breast	115A	Moderate	++	S ++
Carcinoma of ovary	92	Slow	+	R −
Carcinoma of kidney	123	Fast	++++	S +
Hodgkin's disease	61	Fast	+++	R −
Myelogenous leukemia	56	Fast	+++	S ++
Chronic lymphatic leukemia	95	Fast	+++	S ++
Chronic lymphatic leukemia	3B	Fast	+++	±
Lymph node (herniorraphy)	83	Moderate	+++	R −
Lymph node (herniorraphy)	94	Moderate	+++	S +
Lymph node (varicose-vein patient)	93	Moderate	++	S +
Lymph node (herniorraphy)	85	Slow	++	S +
Lymph node (uninvolved node from patient with carcinoma)	91	Fast	+++	S +

*Slow=migration 1 to 100 μ and/or density 10 to 100 cells in 150× fields per 72 hours.
Moderate=migration 100 to 200 μ and/or density 100 to 200 cells in 150× fields per 72 hours.
Fast=migration beyond 200 μ and/or density over 200 cells in 150× fields per 72 hours.

† +=cells migrated less than 150 μ from explant.
++= " " 150 to 200 " " "
+++= " " 200 to 300 " " "
++++= " " 300+ " " "

The damage in each case was evaluated 1 plus (+). In 2 of these, Feulgen-positive material surrounding the nucleoli was seen to be altered. In the other 2 series mitoses were checked. Marked cytotoxicity was never observed.

Cytotoxicity was observed in 2 of 4 sarcoma series. Changes included irreversible granulation and other marked cytotoxic alterations. Two of 4 melanoma series were damaged by actinomycin D. Cells affected were markedly granular and distintegrated or were pyknotic and/or displayed decreased nucleolar size.

A comparison was made between the growth rate and cytotoxic response of all primary cultures of neoplastic and normal tissue (table 2) to determine the amount of correlation. An examination of these two characteristics indicated an absence of correlation between growth and cytotoxic response other than that expected due to chance. Since all primary cultures were handled in an identical manner, regardless of the tissue type, differences in cytotoxic response to actinomycin D apparently were not reflections of either differences in rates of growth or methods of culture.

Cellular Changes in Established Cell Lines

The second phase of this report is concerned with established cell lines and their response to actinomycin D at the same concentrations used previously. These cell lines are HeLa, normal conjunctiva, and normal kidney—the latter two were originally cultured by Chang. Cytological observations of the 3 cell lines treated with 2 concentrations of actinomycin D, *i.e.*, 1×10^{-3} μg. per ml. (8.3×10^{-7} mmole) and 1×10^{-4} μg. per ml. (8.3×10^{-8} mmole) revealed an absence of marked cytotoxicity in all cell lines with reference to pyknosis, cytolysis, loss of staining capacity, granulation, vacuolation, and increased cellular debris. However, mild cytotoxic changes were observed for mitosis. As seen in table 3, the number and percent of normal dividing HeLa, conjunctiva, and kidney cells following 48- and 96-hour treatment with actinomycin D was reduced. This reduction was as much as 60 percent in conjunctiva cell cultures. Differences in the percent of normal metaphases *versus* aberrant metaphases became apparent in all cell lines exposed 96 hours to the drug. There was an increase in the number of condensed or pyknotic metaphases and a decrease in the normal metaphases as the drug concentration increased. The accumulation of aberrant metaphases was greater in conjunctiva and kidney cultures than in HeLa cultures. The cell line least affected by the drug was HeLa. On the other hand, the ratio of dividing cells to resting cells in drug-treated conjunctiva and kidney cell cultures was markedly altered.

Mitotic Changes Produced by Actinomycin D

Differences in response to drug treatment between several primary cultures and the 3 established cell lines became apparent when mitotic counts were compared. No mitoses were observed in 3 primary cultures

TABLE 3.—*Mitotic changes in established cell lines following exposure to actinomycin D*

Cell line	Exposure time (*hours*)	Concentration (*μg./ml.*)	Mitoses (*percent*)	Prophases (*percent*)	Metaphases (*percent*) normal / pyknotic*	Anaphase† (*percent*)	Telophase (*percent*)	Pyknotic cells (*percent*)
HeLa	48	Control	2. 8	16. 0	48. 2 $\frac{21.4}{26.8}$	0	35. 7	0. 006
		1×10^{-4}	4. 1	6. 7	64. 8 $\frac{19.6}{45.2}$	1. 8	26. 5	0. 007
		1×10^{-3}	3. 6	5. 3	70. 0 $\frac{23.4}{47.6}$	1. 3	23. 3	0. 006
	96	Control	2. 8	17. 2	57. 4 $\frac{40.2}{17.2}$	8. 1	17. 2	0
		1×10^{-4}	3. 2	26. 2	48. 5 $\frac{25.2}{23.3}$	6. 7	17. 4	0. 003
		1×10^{-3}	2. 9	13. 4	56. 1 $\frac{16.8}{39.3}$	7. 7	23. 5	0. 004
Human conjunctiva—Chang's cell line	48	Control	6. 6	2. 6	85. 1 $\frac{83.8}{1.3}$	4. 5	7. 7	0. 001
		1×10^{-4}	2. 7	8. 3	82. 1 $\frac{78.6}{3.5}$	2. 4	7. 1	0. 006
		1×10^{-3}	3. 0	2. 0	91. 3 $\frac{91.3}{0}$	2. 1	6. 5	0. 001

	96	Control	2.3	5.4	70.8 $\frac{54.6}{16.2}$	6.9	16.9	0
		1×10^{-4}	3.7	5.4	77.6 $\frac{53.8}{23.8}$	6.1	10.8	0.004
		1×10^{-3}	1.0	8.1	78.2 $\frac{31.0}{47.2}$	8.1	5.5	0.002
Human kidney—Chang's cell line	48	Control	7.6	1.0	94.6 $\frac{90.9}{3.7}$	1.5	3.8	0.001
		1×10^{-4}	4.1	0	79.6 $\frac{57.4}{22.2}$	5.5	14.8	0
		1×10^{-3}	2.3	3.5	96.5 $\frac{63.3}{33.2}$	0	0	0
	96	Control	4.3	4.2	71.8 $\frac{69.0}{2.8}$	8.5	15.4	0
		1×10^{-4}	4.3	2.7	77.6 $\frac{45.9}{31.7}$	6.3	13.5	0
		1×10^{-3}	1.1	0	90.3 $\frac{41.9}{48.4}$	3.2	6.4	0

*For HeLa cells only, includes multipolar mitoses.
†For HeLa cells only, includes telophases and postphases as described by Moorhead and Hsu (*16*).

TABLE 4.—*Mitotic changes in primary cultures following exposure to actinomycin D*

TC# and patient	Diagnosis and tissue	Exposure time (*hours*)	Concentration (*μg./ml.*)	Mitoses (*percent*)	Prophases (*percent*)	Metaphases (*percent*)	Anaphases (*percent*)	Telophases (*percent*)	Pyknotic cells (*percent*)
94 J. K.	Normal inguinal lymph node; reticular cells.	96	Control 1×10^{-4} 1×10^{-3}	1. 3 0 0	7. 6	76. 9	0	15. 5	0
91 M. K.	Normal cervical lymph node;* reticular cells.	96	Control 1×10^{-4} 1×10^{-3}	1. 6 0 0	69	29	0	2	0
95 D. M.	Chronic lymphatic leukemia; axillary lymph node; reticular cells.	96	Control 1×10^{-4} 1×10^{-3}	0. 7 0 0	0	100	0	0	0. 003
123 K. T.	Carcinoma of kidney; node from right nephrectomy scar.	96	Control 1×10^{-4}	5. 4 0. 8	0 12. 5	91 62. 5	0 0	9 25	0 0

*"Normal" node containing no invasive cells removed from patient with carcinoma of thyroid with lung metastases.

exposed to drug (table 4). These were 2 normal lymph nodes and 1 chronic lymphatic leukemia lymph-node series (expts. 94, 91, and 95). A significant sixfold reduction in mitosis was also observed in kidney carcinoma cultures (expt. 123). Mitoses were never reduced to zero in the 3 established cell lines treated in an identical manner.

Discussion

Actinomycin D, at extremely low concentrations, produced a range of cytotoxic changes in a series of primary neoplastic and normal human tissues grown *in vitro*. Severe-to-mild cytotoxic changes were observed in primary cultures. Only mild changes occurred to cells of established cell lines grown in the same culture media. In general, damage to primary cultures was of a nonspecific nature and consisted of over-all granulation, pyknosis, vacuolation, and cell death. These cytotoxic changes occurred in some degree to all cell types in a culture population. In a few culture series, the drug effect was manifested by a reduction in the size of nucleoli or by a loss of the Feulgen-positive nucleolar sheath in cells prepared for this reaction.

The presence or absence of a plasma-embryo-extract clot did not appear to influence the response of a culture to actinomycin D. One melanoma primary-culture series was grown directly on glass, as were established cell lines. Yet cells of the melanoma series were markedly sensitive to the drug while established cell lines were not.

Cell populations of many of the primary neoplastic culture series were often exposed in parallel studies to several other chemotherapeutic agents, namely, triethylenethiophosphoramide, 4-*p*-[bis(2-chloroethyl)amino] phenylbutyric acid (Chlorambucil®), or amethopterin. They varied in their response to these agents. No relationship was found to exist between the sensitivity of these neoplastic cells to actinomycin D and their general sensitivity or resistance to other chemotherapeutic agents. The response in primary cultures was apparently dependent on the individual patient source rather than the pathological cell type. Established cell lines never showed a marked cytotoxic response. It is possible that long-time propagation *in vitro* has in some way modified these cells. Differences in drug response between individual primary cultures and the 3 established cell strains are probably functions of their specific biological properties at the time of exposure. Since the primary culture is closer to the species of origin and apparently responds *in vitro* to a given drug as an individual cell type, it would appear that their use in evaluating potential chemotherapeutic agents to be used *in vivo* warrants further study.

Eagle and Foley obtained 50 percent inhibition of growth of 6 established cell lines with low concentrations of actinomycin D. They observed that cell lines deriving from normal and malignant tissues were more or less equally susceptible to a given agent. Their experimental procedure differs from that described in this report in 3 respects: 1) effective actinomycin D concentrations ranged from 4.2×10^{-6} to 1.25×10^{-5} mmole; 2) cultures were redosed with drug on the 2nd, 4th, 5th, and 6th day and

evaluated on the 5th to 8th day; and 3) amount of actual growth as affected by the agent was determined quantitatively by determination of protein content with the Folin-Ciocalteu reagent. In our experiments, actinomycin D effects were observed with concentrations that ranged from 8.3 $\times 10^{-7}$ to 8.3 $\times 10^{-8}$ mmole. The drug was added to growing cultures and allowed to remain in contact with cells for 4 days when controls and treated cultures were fixed and stained. Recent experiments by Biesele (*17*) indicated a greater number of degenerate cells *in vitro*, following exposure to 1 mmole 6-mercaptopurine, when cultures were redosed during an experiment rather than when they were combined with drug initially and not redosed. It is conceivable, therefore, that redosing might mask subtle differential responses among biological systems *in vitro* to chemical agents.

The mechanism of action of actinomycin D has been studied by Foley (*18*) with the use of specific microbiologic bioassay systems. The data indicate that the drug inhibits pantothenate utilization and suggest an interference with the synthesis and/or biological activity of coenzyme A. In our studies the reduction in nucleolar size in drug-treated cells might be explained by the observations cited by Vincent (*19*) who stated that the nucleolus becomes smaller during drastic catabolic states. Actinomycin D, as a metabolic inhibitor, might therefore produce such a catabolic state.

References

(*1*) Waksman, S. A., and Woodruff, H. B.: Bacteriostatic and bactericidal substances produced by a soil actinomyces. Proc. Soc. Exper. Biol. & Med. 45: 609–614, 1940.

(*2*) Brockmann, H., and Muxfeldt, H.: Die Konstitution des Despeptido-actinomycins. Angew. Chem. 67: 617–618, 1955.

(*3*) ———: Die Synthese des Despeptido-actinomycins. Angew. Chem. 67: 618, 1955.

(*4*) Manaker, R. A., Gregory, F. J., Vining, L. C., and Waksman, S. A.: Actinomycin III. The production and properties of a new actinomycin. Antibiotics Ann. 2: 853–857, 1954–55.

(*5*) Field, J. B., Costa, F., Boryczka, A., and Sekely, L. J.: Experimental evaluation of the anticarcinogenic activity of a new antibiotic, actinomycin C. Antibiotics Ann. 2: 842–852, 1954–55.

(*6*) Hackmann, C.: Experimentelle Untersuchungen über die Wirking von Actinomycin C (HBF 386) bei bösartigen Geschwülsten. Ztschr. Krebsforsch. 58: 607–613, 1952.

(*7*) ———: HBF 386 (Actinomycin C), ein cytostatisch wirksamer Naturstoff. Strahlentherapie 90: 296–300, 1953.

(*8*) Schulte, G.: Erfahrungen mit neuen cytostatischen Mitteln bei Hämbolastosen und Carcinomen und die Abgrenzung ihrer Wirkungen gegen Röntgentherapie. Ztschr. Krebsforsch. 58: 500–503, 1952.

(*9*) Schulte, G., and Lings, H.: Erfahrungen mit neuen zytostatischen Mitteln bei Leukosen und Lymphogranulomatosen und die Abgrenzung ihrer Wirkungen gegen Röntgentherapie. Strahlentherapie 90: 301–306, 1953.

(*10*) Farber, S.: Carcinolytic action of antibiotics: Puromycin and actinomycin D. Am. J. Path 31: 582, 1955.

(*11*) Gregory, F. J., Pugh, L. H., Hata, T., and Thielen, R.: The effect of actinomycin D on experimental ascitic tumors in the mouse. Cancer Res. 16: 985–987, 1956.

(12) FANTINI, A., MOSER, L., WILL, L., PARTRIDGE, R., and HALLIDAY, S. L.: The effect of actinomycin D on several mouse tumors. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 108, 1956.

(13) FARBER, S., MADDOCK, C., and SWAFFIELD, M.: Studies on the carcinolytic and other biological activity of actinomycin D. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 104, 1956.

(14) GOLOMB, F. M., GUMPORT, S. L., and WRIGHT, J. C.: The clinical application of cancer chemotherapeutic agents. Proc. 1st Pan. Am. Cytol. Conf. 1957. In press.

(15) EAGLE, H., and FOLEY, G. E.: The cytotoxic action of carcinolytic agents in tissue culture. Am. J. Med. 21: 739–749, 1956.

(16) MOORHEAD, P. S., and HSU, T. C.: Cytologic studies of HeLa, a strain of human cervical carcinoma. III. Durations and characteristics of the mitotic phases. J. Nat. Cancer Inst. 16: 1047–1066, 1956.

(17) BIESELE, J. J.: Tumor specific cytotoxicity of antitumor agents. Screening Procedures for Experimental Cancer Chemotherapy. Ann. New York Acad. Sc. In press.

(18) FOLEY, G. E.: Preliminary observations on the mechanism of action of actinomycin D in microbiologic systems. Antibiotics Ann. 3: 432–436, 1955–56.

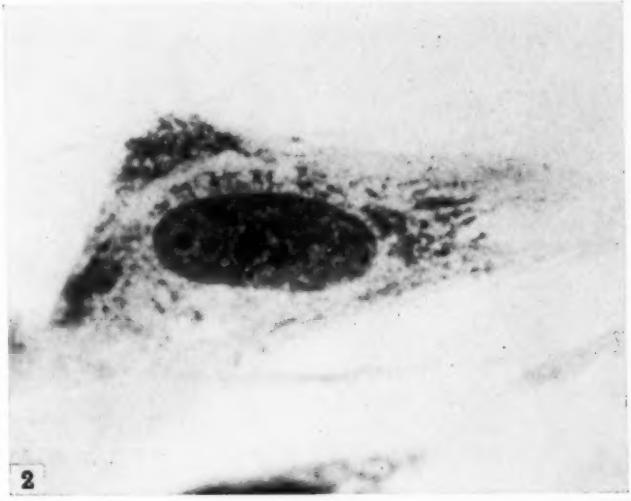
(19) VINCENT, W. S.: Structure and chemistry of nucleoli. Internat. Rev. Cytol. 4: 269–298, 1955.

PLATE 39

FIGURE 1.—Untreated cells in chronic lymphatic leukemia lymph-node tissue culture. Jenner-Giemsa. × 895

FIGURE 2.—Actinomycin D treated cells of same tissue culture series. *Note* reduced nucleolar size. Jenner-Giemsa. × 895

1



2

Effects of Donor and Host Lymphoid and Myeloid Tissue Injections in Lethally X-irradiated Mice Treated with Rat Bone Marrow [1, 2, 3]

GEORGE W. SANTOS [4] *and* LEONARD J. COLE, *Division of Biological and Medical Sciences, U. S. Naval Radiological Defense Laboratory, San Francisco, California*

Summary

J. Nat. Cancer Inst. 21: 279–293, 1958

The effect of injections of isologous mouse or rat spleen, thymus, and bone marrow on the response of lethally X-irradiated LAF_1 mice treated with rat bone marrow was investigated.

The injection of isologous mouse spleen or thymus homogenate on the day of irradiation or 12 days after lethal irradiation with 870 r results in the rapid rejection of the rat bone-marrow graft and death due to bone-marrow failure. No adverse effect upon the survival time of irradiated mice and persistence of the rat bone-marrow graft is noted when isologous liver homogenate, lysed isologous liver, lysed isologous spleen, and lysed isologous thymus are injected. The injection of isologous mouse bone marrow on the day of irradiation or 3 days after irradiation results in the rejection of the rat bone-marrow graft and survival times as long as those seen in irradiated mice treated with isologous bone marrow alone. The survival times of lethally X-irradiated mice treated with bone marrow are significantly shortened when rat spleen or thymus is injected with the rat bone marrow. The rat bone-marrow graft in these mice continues to proliferate until their death. Death results, presumably, from an immunological response of the injected rat cells. No adverse effect either on the graft or on survival time of the host is noted when lysed or lyophilized rat tissue is given with rat bone marrow.

The significance of these findings in elucidating some of the immunological principles involved in foreign bone-marrow transplantation is discussed.

---

[1] Received for publication January 17, 1958.

[2] Presented in part at the "Symposium on Antibodies: Their production and mechanism of action," sponsored by the Biology Division, Oak Ridge National Laboratory, held at Gatlinburg, Tenn., April 8-10, 1957; and in part at the Bone Marrow Transplantation Conference held at New England Deaconness Hospital, Boston, Mass., Oct. 10-11, 1957.

[3] These studies have been supported in part by funds from the Bureau of Medicine and Surgery, U. S. Navy Department, and the Atomic Energy Commission. The opinions and assertions contained herein are not to be construed as official or as reflecting the views of the Navy Department.

[4] Lt. (MC) USNR.

469463—58——6

The protective effect of injection of isologous bone marrow,[5] as well as isologous infant mouse spleen, which contains myeloid elements, in lethally X-irradiated mice is well-established. Animals so treated live for a large fraction of their expected normal life span (*1*). In general, the treatment of lethally X-irradiated mice with homologous or heterologous bone marrow, although "protective," is usually only temporary. Lethally irradiated mice treated with homologous or heterologous bone marrow show, characteristically, after initial protection, a large number of deaths between 30 and 100 days, as contrasted with few deaths, if any, in animals treated with isologous bone marrow (*2*). The pathogenesis of these "late" deaths in homologous and heterologous bone-marrow-treated animals is poorly understood. Three possible mechanisms currently thought to be important in the pathogenesis of these "late" deaths have been clearly restated by Trentin (*2*) as follows:

1) There may be a delayed immunological response of the host to the foreign bone-marrow graft—the so-called homologous or heterologous reaction.

2) There may be a delayed immunological response of the foreign bone-marrow graft to tissues of the host.

3) There may be a failure of the foreign graft to perform all of the vital functions in the host normally performed by autologous or isologous marrow.

The authors have considered for some time that all 3 of the mechanisms mentioned may be operating in producing the "late" deaths. As a means of obtaining further insight into the possible immunological mechanisms involved, we have, in the present study, examined the influence of injected host and donor-type lymphoid and myeloid tissue in LAF_1 mice that have been exposed to an LD100 dose of X radiation and treated with rat bone marrow. The recent report of attempted bone-marrow therapy in humans following therapeutic radiation (*3*) underscores the urgent need for knowledge in this area.

Materials and Methods

Male and female $(C57L \times A)F_1$ mice, 9 to 17 weeks of age, were used in this study as irradiated hosts and as tissue donors. In each experiment up to 40 mice were exposed to a whole-body X-ray dose of 870 r, 250 kvp, measured in air. This dose of X rays is about 100 r above the LD99 (30-day) dose (*4*) and has been uniformly 100 percent lethal for our LAF_1 mice in 15 days.

The radiation factors and details of exposure were the same as reported previously from this laboratory (*5*). The irradiated mice were housed 10 to the cage in metal cages and provided with Purina chow and tap water *ad libitum*. Radiation factors were: 250 kvp X-rays; 15 ma.; 0.5 mm. Cu plus 1 mm. Al filters; HVL, 1.5 mm. Cu; skin-to-target distance 100

[5] Isologous—from same inbred strain.
Homologous—from same species, but different genotype.
Heterologous—from different species.
Autologous—from the same individual or animal.

cm.; dose rate, approximately 25 r per minute, as measured in air with a Victoreen r-meter. Up to 40 mice were irradiated simultaneously at a single whole-body exposure in individual, perforated, lusteroid centrifuge tubes radially positioned around a circular wooden turntable, and rotated at 3.5 r.p.m.

Male and female Sprague-Dawley rats, 8 to 18 weeks of age, were used as bone-marrow and rat-tissue donors. The rat marrow was collected by aspiration, as described previously (*6*), and suspended in cold sterile Tyrode's solution containing 3,200 units of crystalline penicillin G per ml.[6] This suspension was filtered through one layer of surgical gauze. A 0.2 ml. volume of the filtered and chilled bone-marrow suspension (equivalent to 70 mg. of rat bone marrow and containing 640 units of penicillin G) was injected intravenously into nonanesthetized mice 1 to 3 hours after irradiation. Mouse bone marrow was prepared in a similar way but the suspensions of bone marrow were not filtered, and the 0.2 ml. volume of suspension injected contained either 1 or 5 mg. of tissue.

Tissues other than bone marrow (spleen, thymus, and liver) were prepared either in sterile distilled water (lysed material) or in Tyrode's solution (homogenate material) with a Ten Broeck glass homogenizer. Lysate and homogenate suspensions were examined in blood-counting chambers. Lysates were always free of cells on microscopic examination. A few days before the experiment, lyophilizates of tissues were made from homogenate suspended in Tyrode's solution and stored at 5° C. The dried lyophilizate was suspended in distilled water for use. The homogenate, lysate, and lyophilizate were injected intraperitoneally in a 1 ml. volume equivalent to 70 mg. of the respective rat tissue and 3,200 units of added penicillin G. Mouse tissue was prepared and injected in a similar manner except that the equivalent of 5 to 25 mg. of mouse tissue was administered to each animal.

All control animals received the same amount of penicillin in the same volume of Tyrode's solution as animals that were treated with tissue suspensions.

Smears on tail-vein blood were made weekly on most of the mice. The alkaline-phosphatase histochemical technique (*7*) was used for detection of rat granulocytes in the blood smears.

Autopsies were performed on moribund animals. The tissues from each animal were fixed either in 80 percent alcohol for alkaline-phosphatase staining or in formalin for hematoxylin-eosin histological processing.

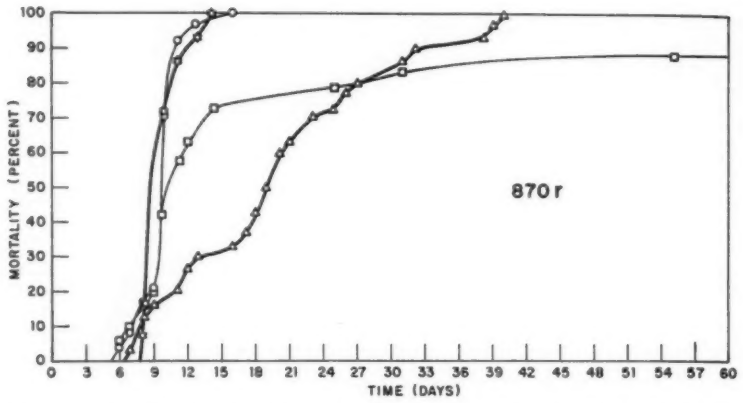
Results

Effect of Injected Host-Type Lymphoid and Myeloid Tissue on Protective Effect and Persistence of Heterologous Bone Marrow

In text-figure 1,[7] 60-day cumulative mortality curves are plotted for 4

[6] Part of the work reported here was completed before our routine use of penicillin, and is so noted where the data are presented.

[7] This is a part of the study completed before our routine use of penicillin.



TEXT-FIGURE 1.—Effect of isologous mouse spleen homogenate injected with rat bone marrow on mortality of lethally irradiated LAF_1 mice: ○ 870 r + 70 mg. rat bone marrow intravenously + 25 mg. isologous mouse spleen homogenate intraperitoneally (*24*); ☆ Tyrode's solution intravenously and intraperitoneally (*14*); △ 870 r + 70 mg. rat bone marrow intravenously + Tyrode's solution intraperitoneally (*30*); □ 870 r + 25 mg. mouse spleen homogenate intraperitoneally + Tyrode's solution intravenously (*19*). Symbols are plotted on the days mice died. Figures in parentheses indicate number of mice.

groups of lethally irradiated (870 r) mice that were given various treatments 2 to 3 hours after irradiation.

The intravenous injection of rat bone marrow alone increases survival time significantly as compared to the irradiated controls. The intraperitoneal injection of isologous adult mouse spleen homogenate alone also has some effect in prolonging survival time, probably because of its small content of myeloid tissue (*8*). However, when an intraperitoneal injection of isologous adult spleen was given with intravenous rat bone marrow, the mortality curve was identical to that of the irradiated controls. Rat granulocytes (alkaline-phosphatase positive cells) were found in the peripheral blood only in those animals treated with rat bone marrow alone. In addition, 2 autopsies of animals at 11 days, which received rat bone marrow with mouse spleen homogenate, showed no rat granulocytes in the bone marrow, spleen, lungs, or liver. Animals injected with rat bone marrow alone after exposure to 870 r routinely show many rat granulocytes in these tissues at 11 days post irradiation.

Cumulative mortalities for 9 groups of irradiated mice that received different types of treatment a few hours after irradiation are recorded in table 1. The data indicate that the injection of isologous mouse thymus homogenate (I) can abrogate the protective action of rat bone marrow like isologous adult mouse spleen homogenate. Isologous mouse thymus lysate (II), isologous mouse spleen lysate (III), isologous mouse liver homogenate (IV), and isologous mouse liver lysate (V) did not appear to modify significantly the protective effect of rat bone marrow during the first 30 days' observation period following irradiation. Rat granulocytes

TABLE 1.—*Effect of injections of isologous tissue homogenates and lysates on mortality of X-irradiated (870 r) mice treated with rat bone marrow*

Group	Number of animals	Treatment*	Cumulative mortality (%)					
			Days following X irradiation:					
			<0	10	15	20	25	30
I	10	70 mg. RBM† intravenously + 5 mg. mouse thymus homogenate intraperitoneally	0	0	90	100	—	—
II	10	70 mg. RBM intravenously + 5 mg. mouse thymus lysate intraperitoneally	0	0	0	20	20	40
III	10	70 mg. RBM intravenously + 5 mg. mouse spleen lysate intraperitoneally	0	0	20	20	40	40
IV	10	70 mg. RBM intravenously + 5 mg. mouse liver homogenate intraperitoneally	0	0	10	30	40	40
V	10	70 mg. RBM intravenously + 5 mg. mouse liver lysate intraperitoneally	0	0	0	0	10	30
VI	10	5 mg. MBM‡ intravenously + Tyrode's solution intraperitoneally	0	0	0	0	0	0
VII	10	70 mg. RBM intravenously + 5 mg. MBM intravenously	0	0	0	0	0	0
VIII	15	70 mg. RBM intravenously + Tyrode's solution intraperitoneally	0	0	7	7	13	27
IX	22	Tyrode's solution intravenously + intraperitoneally	0	0	100	—	—	—

*All treatment groups received penicillin (640 units intravenously and 3,200 units intraperitoneally).
†RBM = Rat bone marrow.
‡MBM = Mouse bone marrow.

were in the peripheral blood at 1 week, and until death in all animals receiving tissue lysates or liver homogenate. Rat cells were present in relatively small numbers at 1 week, but were absent at 14 days and thereafter in all mice that received mouse bone marrow (5 mg.) with rat bone marrow (70 mg.). The best 30-day survival noted was for those animals

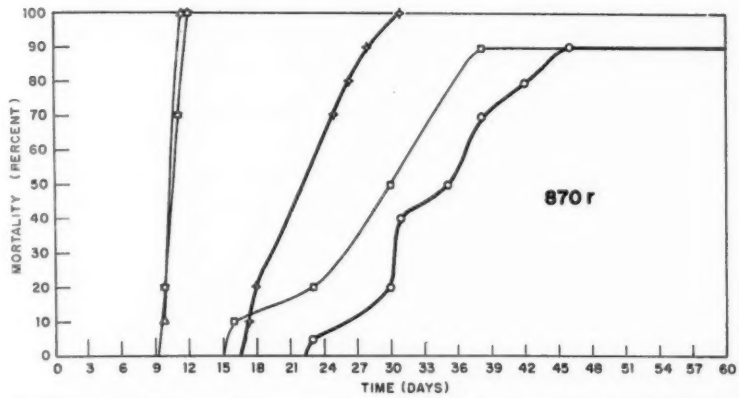
that had received either mouse bone marrow alone (VI) or mouse bone marrow together with rat bone marrow (VII). In animals that received 5 mg. of mouse bone marrow alone (VI), or mouse bone marrow together with rat bone marrow (VII), there was 100 percent survival beyond 60 days. Most animals that died in the 2nd week after thymus or spleen homogenate treatment given with rat bone marrow showed gross evidence of moderate hemorrhagic disease, *i.e.*, petechiae, ecchymoses, and gastrointestinal hemorrhage. These hemorrhagic manifestations were noted only rarely in the other groups in this experiment.

In an additional experiment 10 mice were injected intravenously with 1 mg. of isologous mouse bone marrow a few hours after exposure to 870 r. Fifty percent of them survived beyond 6 months. In another group of 8 mice injected with 70 mg. of rat bone marrow plus 1 mg. of isologous mouse bone marrow, 6 survived beyond 6 months.

Effect of Host-Type Lymphoid and Myeloid Tissue Injected a Few Days after X Irradiation and Rat Bone-Marrow Treatment

Mortality curves (60 days) for 5 groups of irradiated mice (870 r), which received various types of treatment, are plotted in text-figure 2. Mice that were irradiated and given rat bone marrow a few hours after irradiation, followed by mouse spleen homogenate injection *3 days later*, showed a mortality curve essentially identical with that of irradiated controls not given specific therapy. All animals treated with mouse spleen homogenate 3 days after irradiation and initial rat bone-marrow injection showed gross evidence of severe anemia, *i.e.*, watery blood, and hemorrhagic disturbances such as petechiae, ecchymoses, and gastrointestinal hemorrhage. Five autopsies, done at 11 days on the latter animals, revealed a moderate number of rat granulocytes in the bone marrow, but no rat granulocytes were found in spleen, liver, or lung tissues, as contrasted with animals that received mouse spleen homogenate with rat bone marrow a few hours after irradiation in which no rat granulocytes were found. All these animals treated with mouse spleen homogenate showed multiple petechiae and bleeding points in the gut and, in addition, showed subarachnoid collections of large amounts of blood. Hemorrhagic manifestations were not pronounced in the irradiated controls (5 gross autopsies).

When irradiated mice were given mouse spleen homogenate *12 days after* the initial injection of rat bone marrow, shortening of survival time was observed as compared to animals receiving rat bone marrow alone (text-fig. 2). Blood smears were positive for rat granulocytes in all of these animals (spleen-treated group) at 14 days. After this time the number of alkaline-phosphatase positive cells decreased progressively, and in some animals no rat cells were seen in the peripheral blood. Most of these mice showed signs of anemia and hemorrhagic diathesis but no subarachnoid hemorrhages; however, the manifestations of hemorrhagic disease were not as pronounced as those noted in the groups of mice receiving mouse spleen homogenate 3 days after irradiation and initial rat bone-marrow injection. One autopsy at 25 days post irradia-



TEXT-FIGURE 2.—Effect of isologous mouse spleen homogenate and mouse bone marrow, injected a few days post irradiation, on the mortality of lethally X-irradiated LAF_1 mice treated initially (2–3 hours after irradiation) with rat bone marrow and penicillin: ☆ 870 r + Tyrode's solution intravenously and intraperitoneally (10); ○ 870 r + 70 mg. rat bone marrow intravenously + Tyrode's solution intraperitoneally (10); □ 870 r + 70 mg. rat bone marrow intravenously + Tyrode's solution intraperitoneally + 1 mg. of mouse bone marrow intravenously 12 days post irradiation (10); ✦ 870 r + 70 mg. rat bone marrow intravenously + Tyrode's solution intraperitoneally + 5 mg. mouse spleen homogenate 12 days post irradiation (10); △ 870 r + 70 mg. rat bone marrow intravenously + Tyrode's solution intraperitoneally + 5 mg. mouse spleen homogenate 3 days post irradiation (10). All animals received 3,840 units of penicillin 2 to 3 hours after irradiation. Symbols are plotted on the days mice died. Figures in parentheses indicate number of mice.

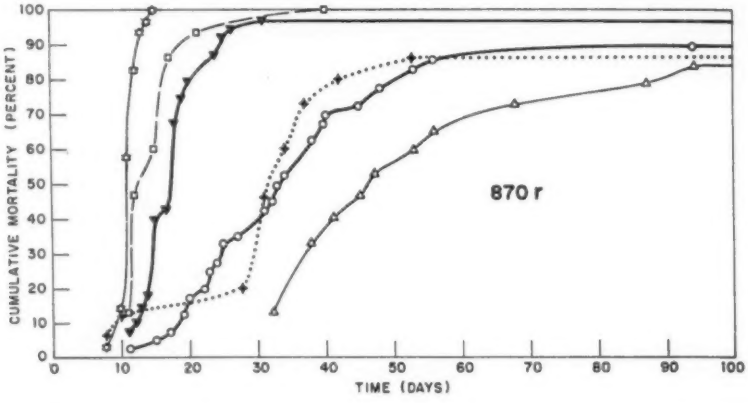
tion showed a few rat granulocytes in the bone marrow, spleen, liver, and lungs, but none in the peripheral blood.

The mortality curve for animals given 1 mg. of mouse bone marrow 12 days after irradiation and rat bone-marrow injection is like that seen after treatment with rat bone marrow alone. Blood smears from this group were positive for rat granulocytes at the 1st week and thereafter. The number of rat cells in these blood smears was no different from that seen in animals given only rat bone-marrow treatment. An autopsy performed at 38 days post irradiation on a moribund animal, which had initially received rat bone marrow and mouse bone marrow 12 days later, showed many rat cells in the peripheral blood, spleen, bone marrow, and liver. The 1 survivor from this group and the 1 survivor of treatment with rat bone marrow alone showed rat granulocytes in the peripheral blood beyond 5 months. There was no evidence of influence by the injected mouse marrow on the rat bone-marrow graft as measured by the relative number of alkaline-phosphatase positive cells in peripheral blood and tissues.

In another experiment, 10 irradiated mice, which were given 1 mg. of mouse bone marrow 3 days after initial rat bone-marrow therapy, showed 50 percent survival beyond 6 months. Rat granulocytes were not seen in the peripheral blood of these mice at 2 weeks and beyond.

Effect of Donor-Type Lymphoid Tissue Injected on the Day of Irradiation

In text-figure 3, the mortality curves of irradiated, rat bone-marrow-injected mice that received either rat spleen homogenate or rat thymus homogenate intraperitoneally are compared to those of irradiated, rat-bone-marrow-injected mice that received either lyophilized or lysed rat spleen intraperitoneally.[8] The mortality data for irradiated controls (no tissue injections) and for irradiated mice that received only rat bone marrow are plotted for comparison. All mice received 3,840 units of penicillin on the day of irradiation.



TEXT-FIGURE 3.—Effect on mortality of rat spleen or rat thymus injections given together with rat bone marrow and penicillin in lethally irradiated mice. ☆ 870 r + Tyrode's solution intravenously and intraperitoneally (28); □ 870 r + 70 mg. rat bone marrow intravenously + 70 mg. rat thymus homogenate intraperitoneally (15); ▼ 870 r + 70 mg. rat bone marrow intravenously + 70 mg. rat spleen homogenate intraperitoneally (40); ○ 870 r + 70 mg. rat bone marrow intravenously + Tyrode's solution intraperitoneally (40); ✦ 870 r + 70 mg. rat bone marrow intravenously + 70 mg. rat spleen lyophilizate intraperitoneally (15); △ 870 r + 70 mg. rat bone marrow intravenously + rat spleen lysate intraperitoneally. All animals received 3,840 units of penicillin 2 to 3 hours after irradiation. Symbols are plotted on the days mice died. Figures in parentheses indicate number of mice.

The injection of rat spleen or thymus homogenate at the same time as that of rat bone marrow, *i.e.*, a few hours after irradiation, decreased the survival times when compared with irradiated mice that received only rat bone marrow. Mortality following lyophilized rat spleen injection given with rat bone-marrow injection did not appear to differ from that of irradiated mice treated with rat bone marrow alone. On the

[8] No lysed or lyophilized control was carried out for rat thymus, rat spleen homogenate, lysate, and lyophilizate. All rat thymus homogenate contained bacteria, but no quantitative differences in these preparations were noted when plate cultures were made.

other hand, lysed rat spleen injected with rat marrow appeared to delay the onset of death when compared to animals receiving only rat bone-marrow injection.

Practically all mice treated with rat bone marrow alone, or in combination with either *rat* spleen or *rat* thymus (lysed, lyophilized, or homogenate), showed many rat granulocytes in the peripheral blood at death, or at 100 days for survivors, with the following exceptions: One survivor of rat marrow-rat spleen homogenate showed no rat granulocytes in the peripheral blood after 34 days; 1 of 2 survivors of rat bone marrow-lyophilized rat spleen revealed rat granulocytes in the peripheral blood at 60 days but none after 70 days. Of 5 irradiated mice that died 12 days after treatment with rat bone marrow and rat spleen or thymus homogenate, all showed many rat granulocytes in the blood, spleen, bone marrow, and liver. This is in contrast to the finding of no rat granulocytes in any tissues in irradiated animals treated with rat bone marrow plus isologous *mouse* spleen homogenate.

The mean survival time and mortality, at 14 days, of 10 mice that received only rat spleen homogenate after irradiation did not differ from that of irradiated controls without tissue treatment. However, when 10 irradiated mice were given both an intraperitoneal injection of 70 mg. of rat spleen homogenate as well as 1 mg. of mouse bone marrow intravenously, there was 100 percent survival at 6 months. Blood smears were never positive for rat cells.

Observations on Late Deaths of Irradiated Mice Receiving Rat Bone Marrow [9]

All mice that were irradiated with 870 r and treated with rat bone marrow showed rat granulocytes in the peripheral blood at 1 week and persistence of rat cells until death (or at least for the first 30 days post irradiation). Before our routine use of penicillin, given only on the day of irradiation, survival to 60 days and beyond was rare in these rat-bone-marrow-treated mice. At present, survivals are 10 to 20 percent at 4 months and beyond. The majority of animals that live to 4 months show rat cells in the peripheral blood. Some few, however, as noted by others (*7*, *9*), do not show rat cells and apparently the autologous bone marrow has started to function.

In animals not given penicillin on the day of irradiation a common finding was that of liver and lung abscesses; occasionally, the liver assumed, grossly, a white nodular and caseous appearance like that seen by Congdon and Urso (*10*) in irradiated LAF_1 mice treated with homologous bone marrow. Invariably, these abscesses showed gram-positive pleomorphic rods. Microscopic examination indicated that many of the liver abscesses were septic infarctions. The abscesses have been very rare in animals given penicillin on the day of irradiation.

[9] Observations made on approximately 100 mice given penicillin on the day of irradiation and 100 mice not given penicillin.

We have observed as did Congdon (*11*) that graying of hair is delayed in animals with rat bone-marrow grafts. We feel, as he does, that delay of graying is related to absence of hair growth. On the other hand, dermatitis, which was prominent in the irradiated, homologous bone-marrow-treated LAF_1 mice of Congdon and Urso (*10*), was an unusual finding in the present studies. Hemorrhagic lesions in the gut like those described by Congdon and Urso (*10*) were observed in about one third of the mice. The most common lesion, however, consisted of multiple pin-point ulcerations in the gastric fundus and pylorus. Results of histological study will be reported at a later date.

Discussion

Effect of Host-Type Spleen and Thymus Tissue

The injection of isologous mouse spleen or thymus homogenate together with rat bone marrow into LAF_1 mice a few hours after lethal irradiation results in the early rejection of the foreign rat bone marrow. These treated animals die like the untreated irradiated controls, presumably of bone-marrow failure, *i.e.*, lack of sufficient hematopoietic tissue. If the injection of isologous spleen homogenate is delayed for 3 or 12 days after irradiation and rat bone-marrow injection, there is evidence of regression or suppression of the rat bone-marrow graft.

In these experiments, the ability to prevent the growth and/or to reject the rat bone-marrow graft appears to be a specific property of the intact isologous spleen and thymus cells as compared to lysed isologous lymphoid tissues or the intact isologous liver cells. At present, the most likely explanation for these observations is that the injected isologous spleen and thymus cells are capable of initiating an immunological response directed against the foreign rat tissues. Such an interpretation seems justified as far as spleen tissue is concerned in view of reports of the *initiation* of antibody formation by homologous and isologous lymph-node cells sensitized to antigen after transfer (*12–14*). Furthermore, Billingham *et al.* (*15*) have shown that tolerance to homologous skin grafting in the mouse, produced by treating the embryo with homologous tissues, can be abrogated by the injection of isologous lymph-node cells from nontolerant mice not treated as embryos. Finally, Trentin (*16*) has shown that "tolerance" to homologous skin grafts induced by lethal irradiation and homologous bone-marrow injection (bone marrow and skin graft from the same strain) can be abolished by the injection of isologous lymph-node or spleen cells from an unirradiated animal of the host strain.

Evidence for antibody production with respect to thymus tissue is meager. Several workers using a variety of methods have failed to note any antibody production by thymus tissue (*13*, *14*, *17–20*). Recently, however, Stoner and Hale (*21*) and Dixon *et al.* (*22*) have reported data suggesting antibody production by transplanted thymus tissue. It should be emphasized that the search has been for the production of anti-

bodies of the Arthus type and not for the delayed tuberculin type of sensitivity.

Effect of Host-Type Bone Marrow

When isologous bone marrow is injected into lethally irradiated mice, together with, or 3 days after, rat bone marrow, one sees a temporary appearance and later disappearance of the rat bone-marrow graft. These animals, in contrast to rat-marrow-injected mice treated with isologous *spleen* or *thymus*, survive as well as irradiated mice given only isologous bone marrow, *i.e.*, with no late deaths. The rejection of the rat cells is most likely due to an immunological response of the nongranulocytic "lymphoid" cells contained in the injected isologous bone marrow. Alternatively, or concomitantly, there may be an immunological response of regenerating autologous lymphoid tissues. Apparently, animals injected with rat bone marrow and isologous mouse marrow survive—despite the rejection of the rat bone-marrow graft—because they possess sufficient isologous myeloid and erythroid tissue.

The ability of relatively massive amounts of isologous bone marrow (8×10^6 cells) to prevent death of LD30 irradiated mice, which have rejected a rat bone-marrow graft and show severe hematopoietic depression, has been previously shown by Congdon *et al.* (*23*). Furthermore, Congdon and Urso (*10*) and Trentin (*24*) have shown that "late" deaths in irradiated mice given *homologous* bone marrow may be prevented if isologous bone marrow is included in the injected cell suspensions.

Effect of Donor-Type Spleen and Thymus Tissue

When rat spleen and thymus cells as homogenates, as opposed to lysed or lyophilized spleen and thymus, are injected together with rat bone marrow into LAF_1 mice a few hours after lethal irradiation, survival times are considerably less than those observed for lethally irradiated mice treated with rat bone marrow alone. Furthermore, under these conditions, there is no evidence of regression or suppression of the rat bone-marrow graft, and death does not appear to be the result of inadequate amounts of hematopoietic tissue; whereas marrow failure is observed in lethally irradiated mice given rat bone marrow and isologous lymphoid tissue. One likely explanation for the observed decrease in survival time of lethally irradiated mice given rat bone marrow with rat lymphoid tissue is that the injected lymphoid cells initiate, or perhaps augment, an immunological reaction against host tissues. However, animals injected with moderate amounts of isologous bone marrow (5 mg.$\sim 5 \times 10^6$ cells) can successfully circumvent the potentially noxious influence of a massive amount of heterologous lymphoid tissue (70 mg.$\sim 70 \times 10^6$ cells). On the other hand, a small amount of isologous bone marrow (1 mg.) injected 12 days after lethal irradiation and initial rat bone-marrow injection (70 mg.) had no apparent beneficial effect upon survival times, or on persistence of the rat marrow cells when compared to irradiated mice which received rat bone marrow only. This suggests that a firmly

established heterologous bone-marrow graft is capable, under certain conditions, of suppressing or compensating for a small inoculum of potentially immunologically active isologous marrow.

Comment: Since this paper was submitted for publication, two reports have come to our attention. The first paper by D. W. Van Bekkum and O. Vos appeared in Supplement 1, volume 50, of the *Journal of Cellular and Comparative Physiology* in 1957, and reported the abrogation of the therapeutic effect of rat bone marrow in irradiated mice with injections of isologous lymph-node or thymus cells. The second paper by E. E. Schwartz, A. C. Upton, and C. C. Congdon was published in volume 96 of the *Proceedings of the Society for Experimental Biology and Medicine* in 1957, and reported similar results when F_1 mice were given parental spleen cells together with parental marrow.

Heterologous and Homologous Disease in Mice [10]

Rat marrow-mouse irradiation chimeras may show circulating rat γ-globulin,[11] and die, in general, without obvious evidence of bone-marrow failure or evidence of rejection or regression of the rat bone-marrow graft. These observations and our findings, that survival times of lethally irradiated mice are decreased (as compared to animals receiving rat bone marrow alone) when rat lymphoid tissue is given along with rat bone marrow, strongly suggest that immunological mechanisms initiated by the donor bone marrow are important in the pathogenesis of *heterologous* disease.

Uphoff (*27*), Trentin (*24*), and Cole *et al.* (*28*) have reached similar conclusions in their studies concerning homologous bone-marrow transplantation, namely, that the immunological activity of the donor marrow is largely responsible for *homologous* disease. In this connection, Ford *et al.* (*29*) have demonstrated by a chromosomal marker technique the presence of donor cells in host lymphoid as well as myeloid tissues following the injection of homologous or heterologous bone marrow. However, the cell types were not identified, and direct evidence for capacity of these donor cells to function immunologically was not obtained.

The opposite view that an immune response of the lethally irradiated mouse is the prime cause of *heterologous* and *homologous* disease is advocated by other workers (*10*, *23*, *30*), but the evidence presented for this hypothesis is less convincing.

Our observation and those of others (*7*, *9*) that a few (the exception rather than the rule) lethally irradiated mice may eventually reject a rat bone-marrow graft, together with the findings of Zalberg *et al.* (*31*) that some lethally irradiated mice may eventually reject both a rat skin and rat marrow graft, indicate the *possibility* that an immunological reaction initiated by the host may, in some cases, contribute to the *heterologous* disease syndrome.

---

[10] Trentin (*24*) has recently suggested these terms to describe the specific complications incident to successful transplantation of heterologous or homologous bone marrow or lymphoid tissue in the "tolerant" host.

[11] Weyzen and Vos (*25*) tested the sera of 15 chimeras; all sera were positive by precipitation with antirat serum for a component traveling with the speed of γ-globulin. Grabar *et al.* (*26*) demonstrated in a number of chimeras tested that all showed γ-globulin only of rat origin, and albumin only of mouse origin.

General Conclusions Concerning Foreign Bone-Marrow Transplantation

From the present experimental data and the discussion, it is, perhaps, not premature to formulate at this time some pertinent biological principles which may, in general, apply to foreign bone-marrow transplants.

The immunological potential of *both* the bone-marrow graft and the host must be taken into account in explaining the pathogenesis of "late deaths" in irradiated animals that have been injected with homologous or heterologous bone marrow.

The potential of the host's immunogenic tissue[12] remaining or regenerating after irradiation [or other radiomimetic treatment (*32*, *33*)] at a given dose, either in the sublethal or lethal range, will, in a large measure, determine whether a foreign bone-marrow graft will "take" and how long it will continue to grow and function. If the host is able to reject a foreign bone-marrow graft and yet survive, it must have sufficient functional autologous (or be given isologous) myeloid and erythroid tissue. On the other hand, since there may be additional loss of autologous myeloid tissue during "violent" immunological reaction, if the amount of autologous myeloid and erythroid tissue that remains after irradiation or other treatment is below a critical level and falls behind the remaining or regenerating immunogenic tissue of the host, then one might *expect a rejection of the bone-marrow graft and death* of the animal from bone-marrow failure. This latter possibility may provide an explanation for the observations of Gengozian and Makinodan (*30*) that LD30 X-irradiated mice will reject a rat bone-marrow graft and show an enhanced mortality. In similar experiments previously reported, early rejection of rat bone-marrow grafts was noted, but without enhanced mortality (*4*). These apparent differences are perhaps best resolved not by postulating that antigenic differences exist between host and donor in the 2 sets of experiments but rather that there are relative differences in response to irradiation in the hematopoietic and immunogenic tissues in the mice used here and those employed by Gengozian and Makinodan. Quite possibly, in mammalian species other than rodents, successful transplantation of homologous bone marrow may not be feasible with X radiation alone, because a 100 percent lethal dose may not adequately suppress host immunogenic systems.

If the early regeneration or remaining amount of host immunogenic tissue is negligible, as we might expect from relatively high radiation doses, the degree of immunogenic potential in the injected bone marrow will determine *not* whether the *graft survives* but rather how long the *host will survive*. Paradoxically, though it appears undesirable to have an excess of immunogenic cells in the foreign bone-marrow graft, the possibility exists that a small amount of immunologically active tissue may be necessary for *long surviving* grafts to suppress any immunogenic tissue of the host that may be beginning to regenerate.

[12] By this term we mean collectively all cells of the host or graft that *are genetically capable* of reacting immunologically, but not necessarily reflected by classical serology or histology, to antigens derived from the donor or host.

References

(1) Cole, L. J., Nowell, P. C., and Ellis, M. E.: Incidence of neoplasms and other late lesions in mice protected against lethal X-ray doses by spleen homogenate. J. Nat. Cancer Inst. 17: 435–445, 1956.

(2) Trentin, J. J.: Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous or heterologous bone marrow. Proc. Soc. Exper. Biol. & Med. 92: 688–693, 1956.

(3) Thomas, E. D., Lochte, H. L., Jr., Lu, W. C., and Ferrebee, J. W.: Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. New England J. Med. 257: 491–496, 1957.

(4) Santos, G. W., Cole, L. J., and Roan, P. L.: The effect of X-ray dose on the protective action and persistence of rat bone marrow in irradiated mice. U. S. Naval Radiological Defense Laboratory Report USNRDL-TR-181, 12 Sept. 1957. Am. J. Physiol., 1958. In press.

(5) Cole, L. J., Fishler, M. C., Ellis, M. E., and Bond, V. P.: Protection of mice against X irradiation by spleen homogenates administered after exposure. Proc. Soc. Exper. Biol. & Med. 80: 112–117, 1952.

(6) Fishler, M. C., Cole, L. J., Bond, V. P., and Milne, W. L.: Therapeutic effect of rat bone-marrow injection in rats exposed to lethal whole-body X radiation. Am. J. Physiol. 177: 236–242, 1954.

(7) Nowell, P. C., Cole, L. J., Habermeyer, J. G., and Roan, P. L.: Growth and continued function of rat marrow cells in X-radiated mice. Cancer Res. 16: 258–261, 1956.

(8) Cole, L. J., and Ellis, M. E.: Age, strain, and species factors in postirradiation protection by spleen homogenates. Am. J. Physiol. 173: 487–494, 1953.

(9) Van Bekkum, D. W., Vos, O., and Weyzen, W. W. H.: Homo et hétérgreffe de tissus hématopoïtiques chez la souris. Rev. hémat 11: 477–485, 1956.

(10) Congdon, C. C., and Urso, I. S.: Homologous bone marrow in the treatment of radiation injury in mice. Am. J. Path. 33: 749–767, 1957.

(11) Congdon, C. C.: Pathologic findings in the delayed heterologous bone marrow reaction. (Abstract.) Rad. Res. 7: 310, 1957.

(12) Harris, T. N., and Harris, S.: The genesis of antibodies. Am. J. Med. 20: 114–132, 1956.

(13) Harris, S., and Harris, T. N.: Studies on the transfer of lymph node cells. V. Transfer of cells incubated *in vitro* with suspensions of Shigella paradysenteriae. J. Immunol. 74: 318–328, 1955.

(14) Harris, T. N., Harris, S., and Farber, M. B.: Studies on the transfer of lymph node cells. VI. Transfer of cells incubated in vitro with Shigella-treated rabbit serum. J. Immunol. 75: 112–122, 1955.

(15) Billingham, R. E., Brent, L., and Medawar, P. B.: Acquired tolerance to skin homografts. Ann. New York Acad. Sc. 59: 409–415, 1955.

(16) Trentin, J. J.: The immunological basis for induced tolerance to skin homografts in irradiated mice receiving bone marrow transfusions. Transpl. Bull. 4: 74–76, 1957.

(17) Harris, T. N., Rhoads, J., and Stokes, J., Jr.: A study of the role of thymus and spleen in the formation of antibodies in the rabbit. J. Immunol. 58: 27–32, 1948.

(18) Bjørneboe, M., and Gormsen, H.: Experimental studies on the role of plasma cells as antibody producers. Acta. path. et microbiol. scandinav. 20: 649–692, 1943.

(19) Fagraeus, A.: Antibody production in relation to the development of plasma cells. Acta. med. scandinav., 130 (supp. 204): 3–122, 1948.

(20) Thorbecke, G. J., and Keuning, F. J.: Antibody formation *in vitro* by haemopoietic organs after subcutaneous and intravenous immunization. J. Immunol. 70: 129–134, 1953.

(21) STONER, R. D., and HALE, W. M.: Antibody production by thymus and Peyer's patches intraocular transplants. J. Immunol. 75: 203–208, 1955.

(22) DIXON, F. J., WEIGLE, W. O., and ROBERTS, J. C.: Comparison of antibody responses associated with the transfer of rabbit lymph-node, peritoneal exudate, and thymus cells. J. Immunol. 78: 56–62, 1957.

(23) CONGDON, C. C., MAKINODAN, T., and GENGOZIAN, N.: Effect of injection of rat bone marrow on reticular tissues of mice exposed to X radiation in the midlethal dose range. J. Nat. Cancer Inst. 18: 603–613, 1957.

(24) TRENTIN, J. J.: Induced tolerance and "homologous disease" in X-irradiated mice protected with homologous bone marrow. Proc. Soc. Exper. Biol. & Med. 96: 139–144, 1957.

(25) WEYZEN, W. W. H., and VOS, O.: Production of rat serum proteins in irradiated mice. Nature, London, 180: 288–289, 1957.

(26) GRABAR, P., COURCON, J., ILBERG, P. L. T., LOUTIT, J. F., and MERRILL, J. P.: Immunologie.–Étude immuno-électrophorétique du sérum de Souris irradiées par des doses létales de rayons X et protégées par des cellules de la moelle osseuse de rats. Compt. rend. Acad. sc. 245: 950–952, 1957.

(27) UPHOFF, D. E.: Genetic factors influencing irradiation protection by bone marrow. I. The F_1 hybrid effect. J. Nat. Cancer Inst. 19: 123–130, 1957.

(28) COLE, L. J., GARVER, R. M., and ELLIS, M. E.: Transfer of donor immunological reactivity to X-irradiated, F_1 hybrid mice by injection of parental bone marrow. Federation Proc. 17: 28, 1958.

(29) FORD, C. E., ILBERG, P. L. T., LOUTIT, J. F.: Further cytological observations on radiation chimeras. J. Cell. & Comp. Physiol. 50: (supp. 1) 109–121, 1957.

(30) GENGOZIAN, N., and MAKINODAN, T.: Antibody response of lethally X-irradiated mice treated with rat bone marrow. J. Immunol. 77: 430–436, 1956.

(31) ZAALLBERG, O. B., VOS, O., and VAN BEKKUM, D. W.: Surviving rat skin grafts in mice. Nature, London, 180: 238–239, 1957.

(32) MAXWELL, R. E., and WESTON, J. K.: Amelioration of myleran-induced bone marrow damage in rats with homologous marrow injections. Federation Proc. 15: 457, 1956.

(33) WESTON, J. K., MAXWELL, R. E., LEE, M., FINZEL, J., and FISKEN, R. A.: Curative effect of rat bone-marrow transfusions in aplastic (severe hypoplastic) anemia in rats induced by myleran, a radiomimetic chemical. Federation Proc. 16: 377, 1957.

Effect of Carcinogenic and Noncarcinogenic Hydrocarbons and Hepatocarcinogens on Rat Liver Regeneration [1]

LEON L. GERSHBEIN,[2] *Biochemistry Research Laboratory, Department of Biology, Illinois Institute of Technology, Chicago, Illinois*

Summary

J. Nat. Cancer Inst. 21: 295–310, 1958

The extent of liver regeneration in partially hepatectomized, adult male rats was determined after subcutaneous injection of various hydrocarbons in peanut oil; observation period was 10.5 to 10.8 days. Daily administration of phenanthrene, anthracene, chrysene, pyrene, acenaphthene, technical picene, 1,2-benzpyrene, and methylnaphthalene oil at over-all dosages ranging up to about 10 mmoles per kg. (7 injections), did not significantly alter the control rate of liver regeneration. But successive injections of carcinogenic hydrocarbons—3-methylcholanthrene, 1,2,5,6-dibenzanthracene, 9,10-dimethyl-1,2-benzanthracene, and 3,4-benzpyrene—at total levels of 0.32 to 0.91 mmole per kg. caused definite increases in extent of liver regeneration; the weak carcinogen 1,2-benzanthracene (0.96 mmole/kg.) proved negative. As exemplified by 9,10-dimethyl-1,2-benzanthracene, a low over-all dosage of 0.19 mmole per kg. was insufficient for a response. Although fewer than 7 injections also caused an increase, an equivalent amount delivered as a single injection on the 3rd day following surgery was ineffective. Repeated injections of the purified noncarcinogenic hydrocarbons, phenanthrene and acenaphthene, led to an elevation in liver regeneration only when the total dose was raised to about 30 mmoles per kg., the resulting activity undoubtedly being due to a direct action of these compounds on the liver. In contrast to carcinogenic hydrocarbons, where an effect was observed on feeding or injecting hepatocarcinogens (p-dimethylaminoazobenzene and 2-acetylaminofluorene), the trend was toward a decrease in extent of liver regeneration. When groups of intact rats were injected with 8 hydrocarbons, alterations in liver-body weight ratios over controls were not always reproducible. No significant liver pathology was observed with any treated group as compared with corresponding controls. The application of these findings as a possible adjunct to the usual laborious methods of carcinogenicity screening is discussed.

[1] Received for publication February 3, 1958.

[2] The author is indebted to Dr. Robert Schrek of the Veterans Administration Hospital, Hines, Ill., for his aid in the microscopic examination of liver sections and to Miss Ruth Zelman for the preparation of the slides.

469463—58——7

In a study of the effect of various hydrocarbons and *p*-dimethylaminoazobenzene[3] and diethylstilbestrol, administered subcutaneously and intraperitoneally, upon the ascorbic acid content of the liver in mice, Kennaway, Kennaway, and Warren (*1*) determined the ratio of liver weight to body weight for representative groups. The ratios for animals injected with a single dose (1 to 40 mg.) of carcinogenic hydrocarbons in peanut or sesame oil, such as 3,4-benzpyrene, cholanthrene, 3-methylcholanthrene, 1,2,5,6-dibenzphenanthrene,[4] 1,2,5,6-dibenzanthracene,[5] and the very weak carcinogen 1,2-benzanthracene,[6] ranged higher than those produced by the oils alone. Naphthalene, anthracene, and phenanthrene were without action. Intraperitoneal injection appeared to be more efficacious than subcutaneous treatment and the results were irregular with the noncarcinogenic hydrocarbons and *p*-dimethylaminoazobenzene. Furthermore, the effects were equivocal or absent in females in which the ratio of liver weight to body weight generally ranges higher than in male mice (*2*). The elevation in liver weight elicited by carcinogenic substances appeared to counteract the increase in hepatic ascorbic acid. No pathological changes were evident in livers from mice treated with 3,4-benzpyrene. It is of interest that with lower single doses of 1,2,5,6-dibenzanthracene (100 mg./kg.) and 3-methylcholanthrene (50 mg./kg.) in olive oil or lard, Boyland and Mawson (*3*) observed that the respective mouse livers underwent no significant change in the levels of ascorbic acid, cholesterol, or glutathione over a 20-day period after intraperitoneal injection; the livers from the 3-methylcholanthrene group appeared to be normal. However, 1 injection of 3,4,5,6-dibenzcarbazole,[7] at a level of 12.5 mg. per kg., caused an increase in hepatic glutathione and extensive bile-duct proliferation occurred prior to this in almost half the mice; with 25 mg. per kg., some liver necrosis was noted in a few of the animals that also suffered decreases in hepatic glutathione content.

Similar to the mouse experiments of Kennaway and coworkers (*1*), Elson and Harris reported increases in liver-body weight ratios for male Wistar rats, weighing 70 to 205 gm., injected intraperitoneally with a single dose of 50 mg. 1,2,5,6-dibenzanthracene in peanut oil (*4*). This occurred for groups of animals fed diets containing either 5 or 20 percent protein and sacrificed over varying periods following injection. One series on the 5 percent protein diet that received 50 mg. pyrene displayed no increase over the control ratio of liver to body weight. The effect with 1,2,5,6-dibenzanthracene did not appear to stem from any elevation in liver fat or water content. An over-all decrease in hepatic deoxypentosenucleic acid (or an increase in the ratio of pentosenucleic to deoxypentosenucleic acids) occurred only in the case of the carcinogen-treated animals.

Enlargement of livers with concomitant fatty degeneration was noted

Chemical Abstracts nomenclature:
[3] *N,N*-Dimethyl-*p*-phenylazoaniline.
[4] Benzo[*c*]chrysene.
[5] Dibenz[*a*, *h*]anthracene.
[6] Benz[*a*]anthracene.
[7] 7 *H*-Dibenzo[*c*, *g*]carbazole.

by Goerner and Goerner in 85 percent of the rats or rabbits repeatedly injected intraperitoneally with a colloidal solution of 1,2,5,6-dibenzan thracene (*5*). Similar results were obtained with some of the rats fed diets containing cholanthrene, benzopyrene, or pyrene at levels of 0.07, 0.10, and 0.20 percent, respectively (*6*). However, gross liver pathology was absent in several on the methylcholanthrene and benzopyrene diets for periods as long as 100 days even though the growth rate was decreased.

The rabbit has also been employed in allied studies. Liver necrosis and cirrhosis resulted in this species on percutaneous application of coal tar or by subcutaneous injection of an ethereal solution of tar (*7*, *8*); shale oil was quite similar in action when introduced intraperitoneally in both rabbits and rats (*9*). With coal tar, the chronic liver alterations, including regeneration, resembled those of typical atrophic cirrhosis. The regenerated tissue that displayed an irregular cellular arrangement, with adenomatous growth occurring in some cases, was still susceptible to the influence of the tar (*8*). In an attempt to correlate the liver changes induced by coal tar or shale oil with prominent component carcinogenic hydrocarbons, Polson investigated two of the outstanding compounds. Whereas 1,2,5,6-dibenzanthracene in paraffin oil caused extreme liver pathology in 60 percent of the rabbits on intraperitoneal treatment, benzopyrene was essentially without effect under comparable conditions. These results were interpreted by Polson as indicative of a lack of parallelism between carcinogenicity and the action on the liver (*10*). Severe hepatic damage with 1,2,5,6-dibenzanthracene dissolved in lard and introduced subcutaneously has also been described by Claude (*11*). Hypertrophy and fatty degeneration of the liver, as well as a decreased vitamin A content and an elevation in total lipide of the hepatic mitochondria, have been demonstrated on intraperitoneal injection of a colloidal solution of this hydrocarbon; *p*-aminoazobenzene proved less active (*5*, *12*).

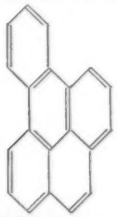
In the present study, a number of noncarcinogenic and carcinogenic hydrocarbons as well as the hepatocarcinogens, *p*-dimethylaminoazobenzene, 4′-fluoro-4-dimethylaminoazobenzene,[8] and 2-acetylaminofluorene,[9] were screened with regard to their effect on liver regeneration in partially hepatectomized rats. Determination of changes in liver-body weight ratios over the controls for groups of animals that had been injected with several hydrocarbons but not operated on was also included.

Materials and Methods

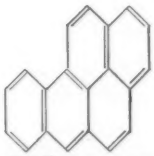
p-Dimethylaminoazobenzene, 3-methylcholanthrene, 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, 9,10-dimethyl-1,2-benzanthracene,[10] 3,4-benzpyrene,[11] and 2-acetylaminofluorene, each of high purity, were purchased from Eastman Kodak Company. The Gesellschaft für Teerverwertung, Germany, comprised the source of the following pure compounds,

Chemical Abstracts nomenclature:
[8] *p*-(*p*-Fluorophenylazo)-*N*,*N*-dimethylaniline.
[9] *N*-(2-Fluorenyl)acetamide.
[10] 7,12-Dimethylbenz[*a*]anthracene.
[11] Benzo[*a*]pyrene.

the respective uncorrected melting points appearing in parentheses: acenaphthene (94 to 96° C.), chrysene (254 to 255° C.), 1,2-benzpyrene[12] (178 to 180° C.), and 3,4-benzpyrene (176 to 178° C.). The latter hydrocarbon and the 3,4-benzpyrene obtained from Eastman Kodak Company



1,2-Benzpyrene



3,4-Benzpyrene

had similar activity in regard to liver regeneration. Technical samples of pyrene and chrysene (tan-colored solid) were furnished by Reilly Tar and Chemical Company. Methylnaphthalene oil, free from tar acid and base, was made available by Koppers Company, Incorporated; it comprised mainly a mixture of the two monomethylnaphthalenes with some dimethylnaphthalenes to the exclusion of benzene and phenols. Technical picene originated from the H. M. Chemical Company. Phenanthrene was purified by treatment of the Eastman product with sodium in accordance with the procedure of Durland and Adkins (*13*) and melted at 97 to 98° C. For the preparation of 4′-fluoro-4-dimethylaminoazobenzene, *p*-fluoroaniline was diazotized and the diazo mixture coupled with dimethylaniline (*14*); the recrystallized solid melted at 153 to 154.5° C.

The respective hydrocarbons and hepatocarcinogens were dissolved in peanut oil (Planter's) so that the injectable volumes ranged under 0.6 ml. except for 1 series that received elevated single doses (1 to 1.4 ml.; *see* table 3). Generally, heating in a water bath was required before injection of the solutions. As chrysene and anthracene were quite low in solubility, higher dilutions with oil and prolonged heating was necessary. Picene and 2-acetylaminofluorene were only slightly soluble so that fine suspensions in peanut oil were used. Diets containing 2-acetylaminofluorene or *p*-dimethylaminoazobenzene were prepared by incorporating the dry powders into ground Purina Fox Chow; all percentages are expressed on a weight basis. For the force feeding of *p*-dimethylaminoazobenzene, the latter was mulled with 20 percent of its weight in gum tragacanth and suspended in water. The mixture was delivered by means of syringe and trocar (daily volume, 2 ml.); the controls received an equivalent amount of water.

Animals.—Young adult males of the Sprague-Dawley strain were employed in the partial hepatectomy series. In the feeding experiments with intact animals, the latter also comprised adult females and immature males of the above strain. The rats were maintained in individual cages, and water and powdered Purina Fox Chow or the special diets were administered *ad libitum*.

Chemical Abstracts nomenclature:
[12] Benzo[*e*]pyrene.

Partially hepatectomized series.—Groups of animals under ether anesthesia were partially hepatectomized by the technique of Higgins and Anderson (*15*). The excised livers were dried to constant-weight in an oven at 100° C. The respective rats were placed on the appropriate diets, force-fed the hepatocarcinogen suspension, or injected subcutaneously with peanut oil or the hydrocarbon-oil mixtures over the specified time intervals. After 10.5 to 10.8 days following surgery, they were sacrificed (ether) and the livers removed and dried to constant-weight. Small sections, stained with hematoxylin and eosin, were reserved for histological examination. As discussed in an earlier report (*16*), the extent of regeneration or the liver increment was calculated from the difference between the dry liver weight at necropsy and the amount of tissue remaining at surgery, which corresponds to 46 percent of the liver weight excised. In most cases, the data from animals suffering weight losses in excess of 10 to 13 percent were discarded.

Series without operation.—Peanut oil (controls) or hydrocarbon-oil mixtures were administered subcutaneously to intact rats and after a period of 10.5 days following the first injection, the animals were anesthetized with ether and the livers removed, drained, and weighed. As in the hepatectomized series, representative tissue sections were also studied microscopically.

Results

Liver weight to body weight ratios expressed on a percentage basis and Fisher *t* values together with body weights for adult females and immature and adult male rats injected subcutaneously with various hydrocarbons in peanut oil are exemplified by the groups listed in table 1. In one series (group A21), single doses of 3-methylcholanthrene and 1,2,5,6-dibenzanthracene (130 to 140 mg./kg.) were administered. Liver increments, *t* values, and body-weight data for partially hepatectomized rats receiving 7 daily subcutaneous injections of each of 13 hydrocarbons in oil, and, for several of the groups at injections that were given at various levels, appear in table 2. In group 4 (9,10-dimethyl-1,2-benzanthracene), injections were carried out on the 1st, 3rd, and 5th days following surgery. The action of a highly purified chrysene sample was also compared with the technical product (group 8). The findings for single subcutaneous doses of 8 of the hydrocarbons are shown in table 3. Data were likewise obtained for partially hepatectomized rats that were administered diets that contained 0.10 and 0.25 percent *p*-dimethylaminoazobenzene and 0.06 and 0.008 percent 2-acetylaminofluorene or were force-fed the first compound daily for 9 days (table 4). The fluorene derivative and 4′-fluoro-4-dimethylaminoazobenzene were also injected subcutaneously daily over a period of 7 days at total dosages of 240 and 290 mg. per kg., respectively.

Microscopic examination of liver sections removed from each of the treated groups revealed no outstanding pathology as compared to the corresponding control tissues.

TABLE 1.—*Liver-weight data for intact rats administered hydrocarbon-peanut oil solutions subcutaneously**

Treatment	Dose		Number of rats	Body weight (*gm.* ± *S. E.*)‡		Wet liver (*gm./100 gm. body weight* ± *S. E.*)	*t*
	Daily (*mg.*)	Over-all (*mg./kg.*)†		Initial	At necropsy		
Group A13 (Females)							
Controls	—	—	13	192 ± 4.4	197 ± 4.1	3.361 ± 0.080	—
Anthracene	12.0	425	14	193 ± 3.8	202 ± 4.5	3.403 ± 0.074	0.39
1,2-Benzanthracene	8.0	290	16	190 ± 4.3	192 ± 3.6	4.019 ± 0.109	5.44§
3-Methylcholanthrene	5.5	210	15	185 ± 4.0	180 ± 3.8	4.162 ± 0.087	6.73§
Group A14 (Males)							
Controls	—	—	16	415 ± 4.5	447 ± 5.6	3.029 ± 0.088	—
Phenanthrene	30–60‖	720	17	402 ± 6.1	427 ± 6.8	3.305 ± 0.080	2.34§
9,10-Dimethyl-1,2-benzanthracene	4.0	68	16	406 ± 5.6	419 ± 5.8	3.348 ± 0.098	2.43§
Group A17 (Males)							
Controls	—	—	15	64 ± 1.3	106 ± 7.0	5.221 ± 0.202	—
Phenanthrene	24.0	2000	15	63 ± 1.8	101 ± 3.7	5.612 ± 0.222	1.30
Chrysene¶	12.0	1100	13	64 ± 2.2	92 ± 2.9	5.970 ± 0.122	3.03§
Pyrene	12.0	1100	12	63 ± 1.5	96 ± 2.6	5.563 ± 0.093	1.89
Anthracene	12.0	1000	13	64 ± 1.4	99 ± 1.5	5.265 ± 0.315	0.12
9,10-Dimethyl-1,2-benzanthracene	1.5	140	12	64 ± 1.5	85 ± 3.7	5.245 ± 0.130	0.10

Group A19 (Females)							
Controls	—	—	14	178 ± 3.6	196 ± 4.1	3.874 ± 0.133	—
Phenanthrene	90.0	3300	11	181 ± 2.9	200 ± 3.4	4.718 ± 0.066	5.21§
Chrysene¶	17.0	650	11	171 ± 3.6	193 ± 3.9	5.000 ± 0.171	5.45§
9,10-Dimethyl-1,2-benzanthracene	1.5	65	12	161 ± 4.7	166 ± 4.8	5.119 ± 0.278	4.24§
Group A21 (Males; single injection)							
Controls	—	—	14	69 ± 1.1	88 ± 1.5	4.501 ± 0.096	—
3-Methylcholanthrene	10.0	140	14	65 ± 1.2	78 ± 2.2	5.936 ± 0.132	8.75§
1,2,5,6-Dibenzanthracene	10.0	130	14	68 ± 1.2	85 ± 2.4	5.905 ± 0.185	6.75§

* Except for group A21, all rats were injected daily for 7 days; sacrificed 10.5 days after the first injection.

† Total dosage administered over the entire test period and calculated on the basis of body weight.

‡ S. E. denotes standard error.

§ $P<0.05$.

|| The dose which was 30 mg. during the first 4 days was increased to 60 mg. for the last 3 injections.

¶ Sample from Reilly Tar and Chemical Corp.

TABLE 2.—*Liver regeneration in partially hepatectomized rats administered hydrocarbons**

Treatment	Dose			Number of rats	Body weight (*gm.* ± *S. E.*)		Liver increment (*gm.* ± *S. E.*)	*t*
	Daily (*mg.*)	Over-all			Initial	At necropsy		
		(*mg./kg.*)	(*mmoles/kg.*)					
Group 1								
Controls	—	—	—	16	249 ± 5. 4	250 ± 5. 6	1. 723 ± 0. 077	—
3-Methylcholanthrene	8. 0	245	. 091	9	251 ± 5. 1	209 ± 6. 1	2. 104 ± 0. 077	3. 23†
1,2,5,6-Dibenzanthracene	5. 0	150	. 054	8	238 ± 3. 1	220 ± 4. 6	2. 065 ± 0. 071	2. 85†
Group 2								
Controls	—	—	—	10	295 ± 4. 1	290 ± 5. 5	1. 930 ± 0. 093	—
3,4-Benzpyrene	6. 0	150	0. 59	7	304 ± 4. 5	263 ± 8. 2	2. 343 ± 0. 168	2. 32†
Group 3								
Controls	—	—	—	14	246 ± 5. 1	260 ± 7. 4	1. 959 ± 0. 083	—
Phenanthrene	6. 0	170	0. 96	14	241 ± 8. 8	251 ± 9. 9	2. 134 ± 0. 088	1. 42
3-Methylcholanthrene	3. 0	85	0. 32	10	244 ± 4. 8	251 ± 8. 8	2. 202 ± 0. 071	2. 21†
Group 4								
Controls	—	—	—	13	270 ± 8. 6	266 ± 8. 8	2. 025 ± 0. 100	—
Anthracene	12. 0	320	1. 8	13	260 ± 4. 4	269 ± 7. 5	2. 210 ± 0. 078	1. 46
Chrysene‡	12. 0	320	1. 4	9	265 ± 6. 0	258 ± 8. 8	2. 257 ± 0. 121	1. 45
Pyrene	12. 0	305	1. 5	13	267 ± 6. 0	285 ± 5. 9	2. 091 ± 0. 111	0. 44
1,2-Benzanthracene	8. 0	220	0. 96	7	261 ± 8. 4	252 ± 9. 5	2. 063 ± 0. 198	0. 20
9,10-Dimethyl-1,2-benzanthracene	15. 0§	170	0. 66	8	265 ± 5. 5	259 ± 10. 4	2. 351 ± 0. 115	2. 38†

Group 5								
Controls	—	—	—	13	243 ± 9.4	247 ± 8.1	1.718 ± 0.138	—
Picene	12.0	345	1.2	13	249 ± 9.2	236 ± 9.6	1.961 ± 0.143	1.47
Methylnaphthalene oil‖	15.0	450	3.2	12	238 ± 9.3	231 ± 10.2	1.517 ± 0.073	1.25
Group 6								
Controls	—	—	—	12	222 ± 6.2	242 ± 5.5	1.653 ± 0.089	—
Phenanthrene	60.0	1800	10.1	9	230 ± 9.8	241 ± 9.7	1.807 ± 0.162	0.96
9,10-Dimethyl-1,2-benzanthracene	1.5	48	0.19	12	224 ± 9.2	219 ± 9.8	1.683 ± 0.081	0.25
Group 7								
Controls	—	—	—	13	244 ± 5.5	251 ± 5.5	1.700 ± 0.079	—
Acenaphthene	25.0	700	4.6	10	247 ± 2.6	255 ± 5.3	1.700 ± 0.074	—
1,2-Benzpyrene	8.0	235	0.93	10	239 ± 6.9	239 ± 7.8	1.780 ± 0.069	0.73
Group 8								
Controls	—	—	—	12	287 ± 7.4	291 ± 7.8	1.965 ± 0.078	—
Phenanthrene	200	5200	29.2	8	279 ± 9.8	260 ± 9.9	2.240 ± 0.077	2.39†
Chrysene¶	100	2400	10.5	7	289 ± 9.0	285 ± 10.1	2.253 ± 0.160	1.81
Chrysene‡	100	2400	10.5	10	290 ± 9.6	295 ± 11.4	2.476 ± 0.248	2.12†
Acenaphthene	200	4900	31.8	11	290 ± 6.8	278 ± 11.1	2.845 ± 0.088	7.46†

*Unless otherwise stated, peanut oil (controls) and the hydrocarbon solutions were injected subcutaneously daily over the first 7 days following surgery.

†$P<0.05$.

‡Technical product from Reilly Tar and Chemical Corp.

§Injected on the 1st, 3rd, and 5th days after surgery.

‖Koppers; free of tar acid and tar base.

¶Highly purified hydrocarbon from Gesellschaft für Teerverwertung, Germany.

TABLE 3.—*Effect of a single subcutaneous injection of various hydrocarbon-oil solutions on liver regeneration (group 15)**

Treatment	Dose			Number of rats	Body weight (*gm.* ± *S. E.*)		Liver increment (*gm.* ± *S. E.*)	*t*†
	Daily (*mg.*)	Over-all			Initial	At necropsy		
		(*mg./kg.*)	(*mmoles/kg.*)					
Controls	—	—	—	14	309 ± 3.3	314 ± 4.1	2.015 ± 0.092	—
Phenanthrene	356	1170	6.6	12	305 ± 5.9	295 ± 6.8	2.108 ± 0.062	0.85
Chrysene‡	228	745	3.3	10	306 ± 5.6	299 ± 5.0	1.940 ± 0.094	0.58
Acenaphthene	308	1040	6.7	12	299 ± 4.0	297 ± 5.0	2.105 ± 0.113	0.64
1,2-Benzanthracene	69	225	1.0	12	307 ± 5.3	296 ± 7.9	1.872 ± 0.092	1.10
9,10-Dimethyl-1,2-benzanthracene	26	85	0.33	12	311 ± 5.5	302 ± 5.9	1.893 ± 0.137	0.72
3-Methylcholanthrene	27	90	0.34	9	304 ± 4.3	297 ± 5.3	2.190 ± 0.094	1.25
1,2,5,6-Dibenzanthracene	28	90	0.32	8	314 ± 7.4	311 ± 7.5	2.136 ± 0.103	0.81
1,2-Benzpyrene	25	80	0.32	11	308 ± 6.6	310 ± 6.9	1.884 ± 0.148	0.77
3,4-Benzpyrene	25	80	0.32	11	302 ± 5.1	292 ± 5.8	2.159 ± 0.109	0.93

* Administered 72 hours following surgery. Volumes of the injectable solutions: 1 to 1.4 ml.

† In all cases, $P>0.05$.

‡ Technical product (Reilly).

TABLE 4.—*Liver regeneration in partially hepatectomized animals administered hepatocarcinogens*

Treatment	Number of rats	Body weight (*gm.* ± *S. E.*)		Liver increment (*gm.* ± *S. E.*)	*t*
		Initial	At necropsy		
Group B3—Diet					
Controls	10	295 ± 7.0	298 ± 7.9	1.972 ± 0.090	—
p-Dimethylaminoazobenzene (0.10%)	11	296 ± 5.0	260 ± 6.1	1.814 ± 0.063	1.45

Group B5—Diet					
Controls	13	293 ± 4.8	288 ± 8.3	1.891 ± 0.082	—
p-Dimethylaminoazobenzene (0.25%)	7	306 ± 4.8	248 ± 7.6	1.236 ± 0.089	5.04*
Group 6—Force Feeding					
Controls	11	246 ± 2.8	243 ± 5.9	1.795 ± 0.064	—
p-Dimethylaminoazobenzene (15.0 mg. daily)†	9	241 ± 4.2	226 ± 8.6	1.758 ± 0.132	0.27
Group B7—Subcutaneous Injection‡					
Controls	14	246 ± 5.1	260 ± 7.4	1.959 ± 0.083	—
4′-Fluoro-4-dimethylaminoazobenzene (10.0 mg. daily)	10	248 ± 4.7	232 ± 9.6	1.831 ± 0.077	1.45
2-Acetylaminofluorene (8.0 mg. daily)	4	247 ± 3.3	223 ± 15.3	1.563 ± 0.123	2.40*
Group B9—Diet					
Controls	16	260 ± 3.6	271 ± 5.8	1.898 ± 0.055	—
2-Acetylaminofluorene (0.06%)	11	271 ± 3.8	234 ± 3.8	1.231 ± 0.076	7.25*
Group B14—Diet					
Controls	10	257 ± 2.4	275 ± 8.7	1.866 ± 0.123	—
2-Acetylaminofluorene (0.008%)	11	255 ± 2.6	241 ± 6.6	1.770 ± 0.068	0.82

*$P < 0.05$.

†Gum tragacanth mull in water; force-fed daily for 9 days starting 24 hours after surgery.

‡Peanut-oil mixtures; 7 daily treatments.

Discussion

Of the noncarcinogenic hydrocarbons administered daily by the subcutaneous route to intact rats, anthracene and pyrene did not significantly alter the control liver-body weight ratio (table 1; over-all dosages up to 1.1 gm./kg.). However, whereas phenanthrene (2.0 gm./kg.) was without effect in immature males, adult rats injected with repeated daily doses, totaling 720 mg. per kg. and 3.3 gm. per kg., respectively, displayed elevations in the ratio of liver weight to body weight. Similar increases were obtained with technical chrysene in adult females and immature males at levels of 650 and 1.1 gm. per kg., respectively, and with the very weakly carcinogenic hydrocarbon, 1,2-benzanthracene (group A13; 290 mg./kg.). Of the carcinogens, successive injections of 3-methylcholanthrene in females at a total dose of 210 mg. per kg. and of 9,10-dimethyl-1,2-benzanthracene in females and adult males (65 to 68 mg./kg.) brought about marked increases in the corresponding liver-body weight ratios. Whereas the last compound was ineffective in immature male rats, increased ratios resulted with such animals given a single injection of a comparable amount of 1,2,5,6-dibenzanthracene as well as 3-methylcholanthrene (group A21).

The above observations with female rats are not in line with those reported by Kennaway, Kennaway, and Warren for mice of this sex (*1*). Although the present study partially corroborates some of their findings with 1,2-benzanthracene and carcinogens and those of Elson and Harris with pyrene and 1,2,5,6-dibenzanthracene in male rats (*4*), irregular results were obtained with the carcinogens as well as the noncarcinogenic hydrocarbons—only sample data of which are advanced in this report. It will be noted that the above workers employed intraperitoneal injection of single doses, while in the present study, the carcinogen-oil mixtures were administered by the subcutaneous route, and, with few exceptions, at repeated doses. This was thought to be a more efficacious procedure, especially with the partially hepatectomized series, and, consequently, was also applied to the intact rat experiments for purposes of comparison.

Greater uniformity in response was achieved by the criterion of liver regeneration in the partially hepatectomized adult male rats. The repeated injection of phenanthrene, anthracene, technical chrysene or pyrene in peanut oil at over-all dosages of 0.96 to 1.8 mmoles per kg. caused no significant change in the extent of liver regeneration over that of the corresponding controls for the 11-day test period (groups 3 and 4, table 2). Methylnaphthalene oil (3.2 mmoles/kg.), acenaphthene (4.6 mmoles/kg.), picene (1.2 mmoles/kg.), and 1,2-benzpyrene (0.93 mmole/kg.) were likewise without effect. When the over-all level of phenanthrene was increased to 10.1 mmoles per kg., the resulting liver increment was little affected, but at a dosage of 29.2 mmoles per kg., a definite increase ensued as was also the case with acenaphthene at a comparably high concentration (31.8 mmoles/kg.; group 8). Although technical chrysene at a total level of 10.5 mmoles per kg. increased the rate of liver regeneration, this was not obtained with the purified hydrocarbon. Of

the compounds mentioned, only chrysene possessed a negligible to very low order of carcinogenicity as reviewed by Hartwell (*17*).

In marked contrast to the negative findings with the noncarcinogenic products at the lower dose ranges, repeated injections of carcinogenic hydrocarbons in partially hepatectomized rats led to striking results. The extent of regeneration was markedly increased with 3-methylcholanthrene at an over-all level of 0.91 mmole per kg. and even with the amount diminished to 0.32 mmole per kg.; 1,2,5,6-dibenzanthracene and 3,4-benzpyrene were also active at doses of 0.54 to 0.59 mmole per kg. (groups 1 to 3, table 2). Furthermore, that fewer than 7 daily injections are effective can be noted from the elevation in liver increment of rats administered 9,10-dimethyl-1,2-benzanthracene in oil on the 1st, 3rd, and 5th days following surgery. Although relatively low over-all levels of the carcinogenic hydrocarbons lead to the effect discussed, presumably a minimal dosage is required. Thus, 9,10-dimethyl-1,2-benzanthracene in amount of 48 mg. or 0.19 mmole per kg. (7 daily injections) did not significantly alter the control average liver increment. The elevation in liver regeneration does not extend to the weak carcinogen, 1,2-benzanthracene injected daily at an over-all dose of 0.96 mmole per kg., though such an amount may augment the liver-body weight ratio in some groups of intact animals, as noted earlier. Because of their purity, it is unlikely that the increase in the extent of liver regeneration engendered by repeated high doses of phananthrene or acenaphthene is due to the presence of carcinogenic moieties but probably stems from a direct effect as such on the liver.[13]

Whereas repeated administration of 3-methylcholanthrene at 0.32 mmole per kg. elicited a marked hepatic response in partially hepatectomized rats, a single injection of this amount on the 3rd day after surgery when the metabolic activity of liver is quite high, proved ineffective (table 3). Similar results were obtained with 9,10-dimethyl-1,2-benzanthracene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene and, as would be expected, with the weakly or noncarcinogenic hydrocarbons, the latter of which were administered at levels up to 6.7 mmoles per kg. Undoubtedly, absorption of the carcinogen over longer periods as afforded by repeated daily subcutaneous injection is of importance with moderate to lower dosages.

Body weights for the intact or partially hepatectomized rats receiving the hydrocarbon solutions were relatively well maintained except possibly for 1 group treated with 3-methylcholanthrene (group 1; 0.91 mmole/kg.; table 2). Furthermore, no discrete alterations over the corresponding control livers could be discerned grossly or microscopically, at least, not during the 11-day observation period. No attempt was made to corroborate the findings of Locatelli who described liver mitochondrial changes that were essentially alike when either carcinogenic or non-

[13] In this connection, preliminary ultraviolet spectrophotometric data for fractions obtained by way of silica gel chromatography of the technical chrysene sample might indicate the presence of small amounts of such carcinogens as 3,4-benzpyrene. In contrast to the purified product, this may account for the results with the higher dosages of the crude material in both the intact and hepatectomized series.

carcinogenic hydrocarbons were injected into intact rats or rabbits, the latter then being sacrificed after periods of 5 days up to 4 months (*18*).

Because of their tumorigenic activity by the oral route, the hepatocarcinogens, *p*-dimethylaminoazobenzene and 2-acetylaminofluorene were administered by diet or force-fed to partially hepatectomized rats. The azo compound proved inactive at a level of 0.10 percent but a very marked decrease in the mean liver increment ensued with the animals receiving the diet containing 0.25 percent of this agent (table 4). Although body-weight losses were especially steep with the latter series, the inhibition in hepatic regeneration probably is not due solely to starvation effects. When even larger amounts of *p*-dimethylaminoazobenzene were introduced, as afforded by force feeding of an aqueous suspension (over-all dosage: 580 mg. or 2.6 mmoles/kg.), the control picture remained unaltered and the respective body weights were relatively well maintained. Undoubtedly, the observed effect on the liver results when the agent is continually ingested over the entire test period instead of daily, single oral doses, a situation which is reminiscent of earlier findings with nicotinic acid or nicotinamide (*19*). With 2-acetylaminofluorene, the diet supplemented with 0.008 percent did not significantly alter the control increment value but when the content was raised to 0.06 percent, a drastic diminution in the regenerative rate was noted and concomitant body-weight losses were not too extreme. The repeated subcutaneous injection of the last compound (total dose: 1.1 mmoles/kg.) was responsible for such excessive decreases in body weight that the data from only 4 of 14 animals could be salvaged. However, possibly a trend toward diminished liver regeneration is also indicated. On the other hand, the injection of 4′-fluoro-4-dimethylaminoazobenzene, a more potent hepatocarcinogen than the parent azo compound (*14*), led to negative results at a total dose of 1.2 mmoles per kg. Thus, in contrast to the behavior of the carcinogenic hydrocarbons, where activity is noted with the hepatocarcinogens, this is in the direction of inhibited liver regeneration. Reference has already been made to the irregular liver-body weight findings of Kennaway and coworkers with *p*-dimethylaminoazobenzene administered to intact mice (*1*).

The increase in liver regeneration in partially hepatectomized animals injected with low doses of carcinogenic hydrocarbons is quite interesting when it is considered that such compounds, particularly, 1,2,5,6-dibenzanthracene, decrease the growth rate in rats and have an inhibitory influence on tumors, especially where low protein diets are administered. Pertinent literature has been reviewed and the findings extended by such groups as Elson and coworkers (*20–22*), among others. Liver succinic dehydrogenase was not significantly lowered under these conditions (*23*).[14]

The present approach by the criterion of liver regeneration may provide an effective tool in the screening of carcinogens, possibly serving as an invaluable adjunct to the laborious painting methods conventionally

[14] It might be pointed out that urease, as well as succinoxidase, was strongly inhibited by metabolic products originating from *p*-dimethylaminoazobenzene (*24*).

employed in this field. Obviously, no claim is made that any agent which promotes an elevation in the regeneration comprises a carcinogen. The above applications must await the testing of additional hydrocarbons and for a better understanding of the hepatic hyperplasia induced by these agents, histochemical, enzymatic, and nucleic acid studies might be instituted. The latter will entail a consideration of alterations produced by the hydrocarbons and superimposed on those due to the regenerating liver as such.

References

(1) KENNAWAY, E. L., KENNAWAY, N. M., and WARREN, F. L.: The effect of aromatic compounds upon the ascorbic acid content of the liver in mice. Cancer Res. 4: 367–376, 1944.

(2) KENNAWAY, E. L., and KENNAWAY, N. M.: The ascorbic acid content of the liver in mice. Cancer Res. 4: 245–250, 1944.

(3) BOYLAND, E., and MAWSON, E. H.: Changes in the livers of mice after administration of 3:4:5:6-dibenzcarbazole. Biochem. J. 32: 1460–1466, 1938.

(4) ELSON, L. A., and HARRIS, R. J. C.: The influence of 1:2:5:6-dibenzanthracene on the nucleic acid content of the liver of rats maintained on high and low protein diets. Brit. J. Cancer 1: 327–334, 1947.

(5) GOERNER, A., and GOERNER, M. M.: The effect of dibenzanthracene on the liver. Am. J. Cancer 37: 518–520, 1939.

(6) WHITE, J., and WHITE, A.: Inhibition of growth of the rat by oral administration of methylcholanthrene, benzpyrene, or pyrene, and the effects of various dietary supplements. J. Biol. Chem. 131: 149–161, 1939.

(7) DAVIDSON, J.: The effect produced on the liver by application of coal tar to animals. J. Path. & Bact. 26: 127, 1923.

(8) ———: Liver necrosis and cirrhosis produced experimentally by coal tar. J. Path. & Bact. 28: 621–626, 1925.

(9) POLSON, C. J.: Experimental liver necrosis from shale oil. J. Path. & Bact. 34: 5–12, 1931.

(10) ———: Experimental liver necrosis produced by 1:2:5:6-dibenzanthracene. J. Path. & Bact. 42: 537–538, 1936.

(11) CLAUDE, A.: Liver degeneration and cirrhosis produced by 1:2:5:6-dibenzanthracene. Am. J. Cancer 31: 100–103, 1937.

(12) GOERNER, A. Effect of dibenzanthracene on vitamin A and total lipid of mitochondria. J. Biol. Chem. 122: 529–538, 1938.

(13) DURLAND, J. R., and ADKINS, H.: Hydrogenation of phenanthrene. J. Am. Chem. Soc. 59: 135–137, 1937.

(14) MILLER, J. A., SAPP, R. W., and MILLER, E. C.: The carcinogenic activities of certain halogen derivatives of 4-dimethylaminoazobenzene in the rat. Cancer Res. 9: 652–660, 1949.

(15) HIGGINS, G. M., and ANDERSON, R. M.: Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch. Path. 12: 186–202, 1931.

(16) GERSHBEIN, L. L., and LABOW, J. A.: Effect of various sulfur compounds on rat liver regeneration. Am. J. Physiol. 173: 55–57, 1953.

(17) HARTWELL, J. L.: Survey of Compounds which Have Been Tested for Carcinogenic Activity. Pub. Health Serv. Publ. No. 149, 2nd ed. Washington, D. C., U. S. Gov't. Print. Office, 1951, pp. 1–583.

(18) LOCATELLI, P.: L'azione di alcuni idrocarburi oncogeni o inattivi sul condrioma delle cellule epatiche. Tumori 21: 122–129, 1947.

(19) GERSHBEIN, L. L.: Effect of nicotinic acid and derivatives on rat liver regeneration. Am. J. Physiol. 184: 47–51, 1956.

(*20*) Elson, L. A., Goulden, F., and Warren, F. L.: The absence of a direct relation between growth inhibition and sulfur metabolism after 1:2:5:6-dibenzanthracene. Brit. J. Cancer 1: 80–86, 1947.

(*21*) Elson, L. A., and Warren, F. L.: The relation between growth inhibition, toxicity and protein metabolism in rats treated with 1:2:5:6-dibenzanthracene. Brit. J. Cancer 1: 86–97, 1947.

(*22*) Elson, L. A., and Haddow, A.: The inhibitory action of 1:2:5:6-dibenzanthracene on the growth of the Walker carcinoma 256 in rats maintained on high and low protein diets. Brit. J. Cancer 1: 97–101, 1947.

(*23*) Elson, L. A., Kennaway, E. L., and Tipler, M. M.: The effect of 1:2:5:6-dibenzanthracene on the ascorbic acid content of the liver of rats maintained on high- and low-protein diets. Brit. J. Cancer 3: 148–156, 1949.

(*24*) Elson, L. A., and Hoch-Ligeti, C.: Inhibition of urease and succinic oxidase by metabolic products of *p*-dimethylaminoazobenzene and by some related amines. Biochem. J. 40: 380–391, 1946.

Endotoxin Treatment and X Irradiation in Mice Bearing Transplanted Tumors [1]

JOANNE W. HOLLCROFT *and* WILLIE W. SMITH,[2]
Radiation Branch, National Cancer Institute,[3] *Bethesda, Maryland*

Summary

J. Nat. Cancer Inst. 21: 311–329, 1958

Salmonella typhosa endotoxin had a small inhibitory effect on the local growth of a lymphosarcoma. X irradiation with 475 r whole-body or 1000 r (200 kv.) to the tumor alone produced the same degree of regression and, in both cases, when endotoxin was given 24 hours before irradiation an additive effect on tumor regression was seen.

Whole-body irradiation of mice bearing a disseminated reticulum-cell sarcoma increased their survival time, but increasing the dose from 600 r to 1500 r (2.5 Mev) did not yield a steadily increasing survival time. Irradiation repeated at intervals of 2 weeks or more was more effective than single exposures. Endotoxin given 24 hours before irradiation increased tolerance to radiation. Endotoxin also produced an increase in survival time of the irradiated tumor-bearing mice which was greater than the sum of the increase attributable to endotoxin or radiation alone.

The necrotizing effect of bacterial lipopolysaccharides on tumors was described a number of years ago by Shear and others (*1–3*). Recently, it has been shown that small amounts of these substances reduce radiation lethality (*4*) by inducing an advanced recovery of hematopoiesis (*5*). The dose that was effective in reducing radiation lethality had only a slight retarding action upon the lymphosarcoma and reticulum-cell sarcoma used in the present experiments. This small dose of endotoxin was used to determine whether or not the endotoxin would sensitize the tumor to radiation and to find the limit of whole-body irradiation that the endotoxin would enable the tumor-bearing animal to tolerate. In addition, a study was made of the dose response of the reticulum-cell sarcoma to whole-body irradiation when the tolerated exposure was extended by endotoxin treatment, by injection of bone marrow from donor mice, and by repeated exposures.

[1] Received for publication February 4, 1958.
[2] With the assistance of Ilo M. Alderman, Bruce L. Weed, Ruth E. Gillespie, and Marion M. Matthews.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

469463—58——8

Materials and Methods

Lymphosarcoma

The fourth and fifth generations of a lymphosarcoma (K-4) were used for evaluation of the effect of an injection of *Salmonella typhosa* endotoxin[4] (*6–9*) upon radiation-induced tumor regression. This tumor arose spontaneously in a (BALB/c × DBA/2)F_1 female. It grows locally to a large size but metastasizes to the lymph nodes, thymus, spleen, liver, kidneys, and lungs.

Experiment 1.—The tumor was transplanted by trocar into 68 (BALB/c × DBA/2)F_1 females 2 months old. On the 40th day they were placed in individual cages and half of them were injected intraperitoneally with 10 μg. of *S. typhosa* endotoxin. Twenty-four hours later, 10 of the treated and 10 of the untreated mice were placed in lead shields with the tumor exteriorized and irradiated with 1000 r. Twelve treated and 12 untreated mice were given 475 r to the whole body. Three additional groups of 12 served as tumor controls, endotoxin controls, and irradiated controls, respectively. Tumor growth was measured with calipers.

Radiation was delivered from 2 opposing Westinghouse Quadracondex tubes, each operating at 200 kv., 15 ma., and filtered with 0.25 mm. Cu and 1 mm. Al. For the tumor exposure, the target distance was 25 cm. and dose rate in air 628 r per minute, while for whole-body irradiation the target distance was 54 cm. and dose rate 123 r per minute.

Experiment 2.—Seventy mice of the same age, sex, and strain were inoculated intravenously with a suspension containing approximately 4×10^6 K-4 lymphosarcoma cells. On the 24th day, half the mice were injected *intraperitoneally* with 10 μg. of the endotoxin, and 24 hours later 18 treated and 17 untreated mice were given 475 r to the whole body under the same conditions as in experiment 1.

Reticulum-Cell Sarcoma

For studies of the endotoxin effect upon the survival of tumor-bearing mice given whole-body irradiation, a reticulum-cell sarcoma, H6867, proved to be more satisfactory than the lymphosarcoma. This tumor also arose spontaneously in a (BALB/c × DBA/2)F_1 female and was carried by serial subcutaneous transplants. It is typical of reticulum-cell sarcomas found in mice. The tumor invades the liver and spleen and sometimes is seen in lung, bone marrow, and circulating blood. The local growth remains small. Transplants of the tenth through the sixteenth generation were made by trocar into (BALB/c × DBA/2)F_1 females 3 months of age. Prior to treatment the tumor-bearing mice and nontumorous controls were placed in individual cages. Endotoxin was given as an *intraperitoneal* injection of 10 μg. of the *S. typhosa* lipopolysaccharide 24 hours before irradiation. Bone marrow was given as an *intravenous* injection of an aliquot of marrow from 2 femurs and 2

[4] We are indebted to Dr. Maurice Landy, of this Institute, for the endotoxin.

tibias of 2- to 3-month-old mice of the same strain. In the section "Results," groups receiving endotoxin are designated E; marrow, M; irradiation, R; and no treatment, C.

Experiments with the reticulum-cell sarcoma are outlined in table 1. In these experiments the mice were given whole-body irradiation from a Van de Graaff generator operating at 2.5 MVCP, 0.6 ma., HVL 1 cm. lead, target skin distance (TSD) 1 meter, and dose rate 250 to 300 r per minute.[5] All doses were delivered as single exposures except the 1500 r dose, which was delivered in 3 exposures of 500 r each on the same day, 2 hours apart or on 3 successive days. Exposures were repeated at intervals of 2 weeks or more in several groups of experiments 4, 6, and 7.

TABLE 1.—*Outline of experiments with reticulum-cell sarcoma—control groups, studied for comparison with each treatment, are not shown*

Expt. No.	Dose (r)	Number of mice per group	Treatment
3	700, 800	10–19	Endotoxin
4	600, 700	10–15	Endotoxin (10 and 50 μg., 10 and 20 days); repeated irradiation
5	1100	10–20	Marrow; endotoxin
6	1000, 1500	20	Marrow; repeated irradiation
7	500	20	Endotoxin; repeated irradiation
8	700	24	Endotoxin; serial killing

Results

Lymphosarcoma

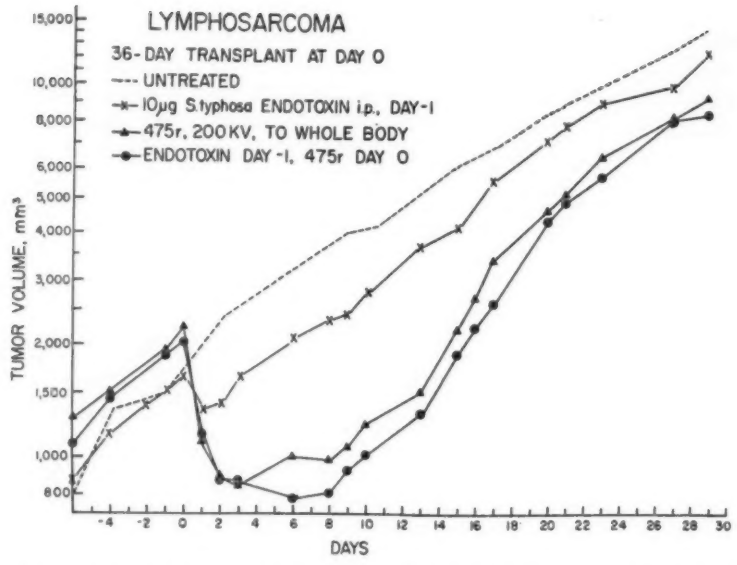
The lymphosarcoma showed about the same degree of regression in mice given 475 r (200 kv.) to the whole body as in mice given 1000 r to the tumor alone (expt. 1, text-figs. 1 and 2). Ten μg. of the *S. typhosa* endotoxin produced only a slight reduction in tumor size. The difference between endotoxin-treated and untreated mice in the irradiated groups was also slight or insignificant, indicating that the endotoxin did not sensitize the tumor to radiation.

The regression of this tumor due to irradiation did not prolong survival. Mean survival times in all groups ranged from 85 to 98 days after transplantation. Irradiation, with or without endotoxin pretreatment, again failed to prolong survival in experiment 2 where the tumor cells were given intravenously. The mean survival times of the 4 groups of tumor-bearing mice (control, endotoxin, 475 r, and endotoxin followed by 475 r) varied between 61 and 64 days.

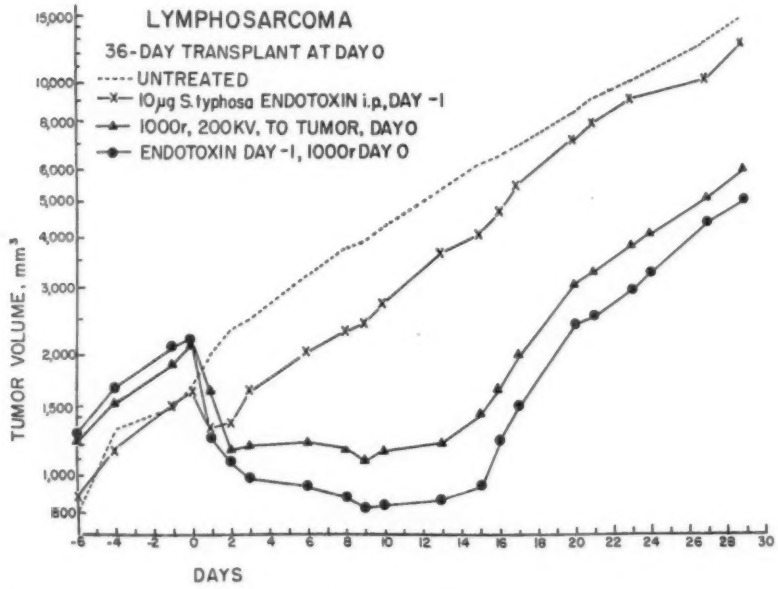
Reticulum-Cell Sarcoma

Under four headings in this section experiments are described in which: the principal effect of the endotoxin treatment was to prevent radiation death (expts. 3 and 5); radiation deaths were negligible and endotoxin

[5] For irradiation and dosimetry with the Van de Graaff generator we are indebted to Dr. Howard L. Andrews.



TEXT-FIGURE 1.—Growth of lymphosarcoma, K-4, following whole-body irradiation, endotoxin, or a combination of both. Irradiation took place on day 0.



TEXT-FIGURE 2.—Growth of lymphosarcoma, K-4, following local irradiation, endotoxin, or a combination of both. Irradiation took place on day 0.

influenced the time of death due to the tumor (expts. 4, 5, and 6); survival was extended by repeated exposures to radiation, with or without prior endotoxin treatment (expts. 4, 6, and 7); and organ and body weights were studied (expt. 8).

The uniformity of survival time within a treatment group in mice bearing the reticulum-cell sarcoma was such that differences as great as 4 days between 2 means in the same experiment generally had a P value no greater than 0.05. The series consisted of 5 experiments with a total of 31 treatment groups averaging 15 mice each.[6] Although mean survival times ranged from 32 to 96 days, the average standard error was only 1.2 days or 2 percent of the mean. It should be noted, however, that survival times might vary considerably from one experiment to another. In the first experiment of this series (expt. 3, table 2) untreated mice died in 32 $\pm$ 0.4 days, while in the remaining experiments the survival times of untreated groups were homogeneous: 43 $\pm$ 0.8, 45 $\pm$ 0.6, 45 $\pm$ 1.3, and 47 $\pm$ 1.9 days. In the latter experiments survival times of treated groups can be compared on almost the same basis as if they were part of the same experiment.

TABLE 2.—*Effect of an injection of* S. typhosa *endotoxin (E) 24 hours prior to irradiation (R) on survival of mice bearing a reticulum-cell sarcoma (T): Irradiation 20 days after transplantation (expt. 3)*

R	Fraction survival more than 3 weeks			Mean survival time *	
	No T	T (*no E*)	TE	T (*no E*)	TE
0		19/19	19/19	32	33
700 r	18/19	5/11	10/10	42	47
800 r	25/28	2/20	19/20	47	47

*Days after transplantation of tumor. Includes only mice that survived longer than 3 weeks after irradiation.

Radiation Death and Its Prevention by Endotoxin Pretreatment

The potential benefit of irradiation in tumor-bearing mice may be offset by their inability to tolerate radiation injury. The predominant effect of the endotoxin injection (expt. 3, table 2) was to prevent acute radiation death in the tumor-bearing mice. While less than 10 percent of the irradiated controls died within 3 weeks of exposure, more than half the irradiated tumor-bearing mice died in that time. In these animals the liver at death was very small (0.6 $\pm$ 0.02 gm.) compared to untreated mice with tumors (5.1 $\pm$ 0.1 gm.) or normal mice (1.1 gm.). The difference in gross appearance of irradiated and nonirradiated tumor-bearing mice is seen in figures 1 and 2. On this evidence we assumed that radiation was primarily responsible for death when it occurred within 3 weeks, and such animals were omitted from calculations of survival time.

When the tumor-bearing mice were pretreated with endotoxin, only 1 of 30 died within 3 weeks of exposure to 700 or 800 r. An even more

[6] The 2 groups with 5 and 2 survivors shown in table 2 are excluded, as well as individual animals that died within 3 weeks of irradiation, as indicated in the tables.

impressive example of this effect was seen in experiment 5 where all tumor-bearing mice exposed to 1100 r died in 10 ± 0.7 days, while 4 of 10 pretreated with endotoxin lived 44 to 55 days after irradiation (65 to 76 days after tumor transplantation).

An advance in hematopoietic recovery is apparently responsible for the increased survival in normal mice given an endotoxin injection prior to irradiation (*4*, *5*). This phenomenon also occurred in the tumor-bearing mice. Table 3 shows granulocyte counts and hemoglobin concentrations in mice given an injection of endotoxin prior to irradiation with 700 r (expt. 4). Granulocyte recovery was already evident on the 6th day, an advance of approximately 11 days over the group given no endotoxin. Also, the severe anemia seen in untreated mice did not develop in the mice pretreated with endotoxin.

TABLE 3.—*Granulocytes and hemoglobin in mice given 700 r on the 21st day after reticulum-cell sarcoma transplantation, without and with an injection of* S. typhosa *endotoxin 24 hours before irradiation (expt. 4)*

Days after irradiation	Granulocytes per mm.³		Hemoglobin gm. per 100 ml.	
	No endotoxin	Endotoxin	No endotoxin	Endotoxin
6	510	1250	11. 4	12. 6
8	560	2040	10. 3	10. 9
10	490	4080	9. 0	11. 6
14	500	3950	7. 4	12. 7
21	4040	4540	12. 3	12. 9
No irradiation.*	5770 ± 610		12. 9 ± 0. 2	

*Tumor-bearing controls, 35 determinations made 21 to 35 days after transplantation. At 42 days many of these had very high leukocyte counts.

Effect of Radiation and Endotoxin on Time of Death Due to the Tumor

That irradiation increased the survival time of mice bearing the reticulum-cell sarcoma is evident from the results shown in table 2. Increasing the radiation dose beyond the limits normally tolerated did not, however, insure a continuing lengthening of the survival time. From the data in table 4 the gain in survival time between 600 and 1000 r was 6 days while the estimated gain for a further dose increase to 1400 r was only half that amount.

Endotoxin increased the survival time of irradiated tumor-bearing mice in cases where the gain was obviously not due to protection against the acute radiation death. The 3 groups of table 4 in which endotoxin was given prior to irradiation showed increased survival times of 6 to 10 days (10 to 16%) over corresponding groups given no endotoxin. The increase in survival time of 27 nonirradiated mice bearing the tumor was only 1.2 days (3.5%).

We attempted to improve this gain by injecting 50 μg. rather than 10 μg. of the endotoxin (700 r, expt. 4). These mice lived no longer, however, than those given the 10 μg. injection. The possibility that earlier

TABLE 4.—*Effect of irradiation with bone-marrow (M) or without a subsequent injection and with* S. typhosa *endotoxin (E) or without endotoxin pretreatment on survival time of mice bearing a reticulum-cell sarcoma: Irradiation 20 to 22 days after transplantation; C=not irradiated; R=irradiated*

Expt. No.	Dose (*r*)	No endotoxin			Endotoxin		Percent gain	
		Three-week survivors	Mean survival time*		Three-week survivors	Survival time* ER	R *vs.* C	ER *vs.* C
			C	R				
4	600	15/15	43	56	15/15	63	30	45
	700	15/15		58	15/15	64	35	48
6	1000 (M)	17/20	45	62			38	
5	1100 (M)	14/19	45	64	15/20	74	42	64
6	1500 (M)†	17/20	45	64			42	
	1500 (M)‡	18/20		66			47	

*Including only the mice that survived longer than 3 weeks after irradiation.
†Three exposures of 500 r each at 9 a.m., 11 a.m., and 1 p.m. followed by a bone-marrow injection.
‡Three exposures of 500 r each on days 20, 21, and 22, the last exposure followed by a bone-marrow injection.

treatment would be more effective was also tested in groups given 10 or 50 μg. on the 9th day and 700 r or no irradiation on the 10th day. Survival times were not improved either by the earlier irradiation or by the earlier endotoxin injection in nonirradiated groups. The combined treatments were somewhat less effective when given 9 to 10 days than 19 to 20 days after transplantation: for 10 μg., 58 compared to 64 days; for 50 μg., 60 compared to 64 days.

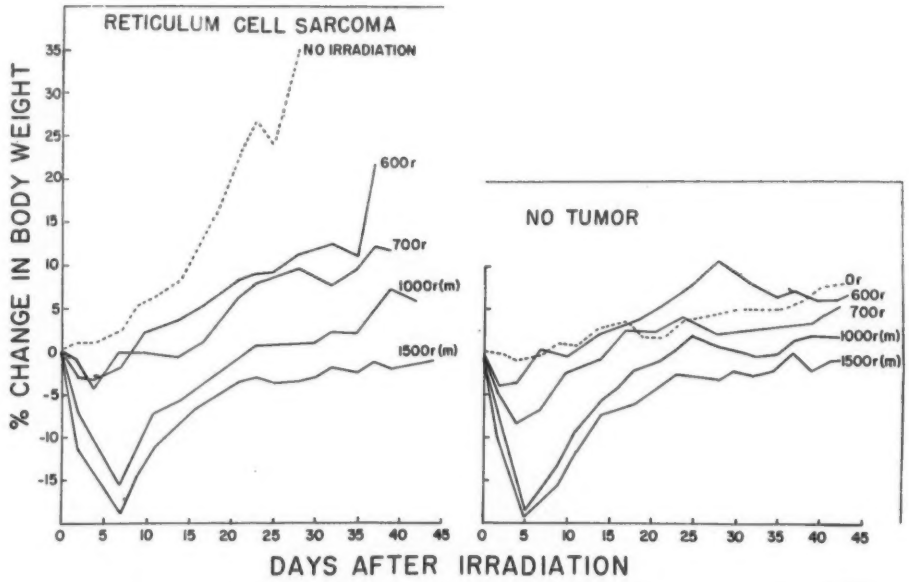
Effect of Repeated Irradiation and Endotoxin Treatment on Time of Death

Radiation rather than tumor was responsible for many deaths in the groups given repeated exposures (table 5). The second exposure to 1000 r, 1500 r in 3 days, and 1500 r in 1 day (expt. 6) was followed by death of 6 in 10, 1 in 8, and 6 in 10 control mice, respectively. Among controls exposed to 500 r 5 times (expt. 7), 19 of 26 given endotoxin and 21 of 26 not given endotoxin died in 80 to 116 days after the beginning of the experiment. Repeated exposures did, nevertheless, prolong the survival time of tumor-bearing mice beyond that obtained with a single exposure. It should be noted that repetition of the 1500 r dose given in 3 days resulted in a longer mean survival time than repetition of the 1500 r dose given in a single day.

The administration of endotoxin before each of the exposures to 500 r (repeated 5 times at intervals of 2 weeks) increased the mean survival time of tumor-bearing mice from 86 ± 1.2 days to 96 ± 2.6 days. Endotoxin failed to prevent radiation deaths in normal mice exposed on this schedule.

Body and Organ Weights

Body weights of mice with the reticulum-cell sarcoma and their controls are shown in text-figure 3. Untreated mice bearing the tumor



TEXT-FIGURE 3.—Body-weight changes of mice bearing reticulum-cell sarcoma, H6867.

TABLE 5.—*Effect of repeated irradiation on survival time of mice with reticulum-cell sarcoma—first exposure 20 or 21 days after transplantation: irradiated (R), non-irradiated control (C), bone marrow after (M), endotoxin before each irradiation (E)*

Expt. No.	Irradiation			Survivors at last exposure*	Mean survival time (*days*)		Percent gain
	Dose (*r*) per exposure	Number of exposures	Days between		C	R	
4	600	2	9	14/14	43	70	63
6	1000 (M)	2	34	18/19	45	71	58
	1500 (M)†	2	36	16/20		72	60
	1500 (M)‡	2	36	18/20		87	93
7	500	5	14	22/24	47	86	83
	500 (E)	5	14	22/24		96	104

*For survival of nontumor controls *see* text.

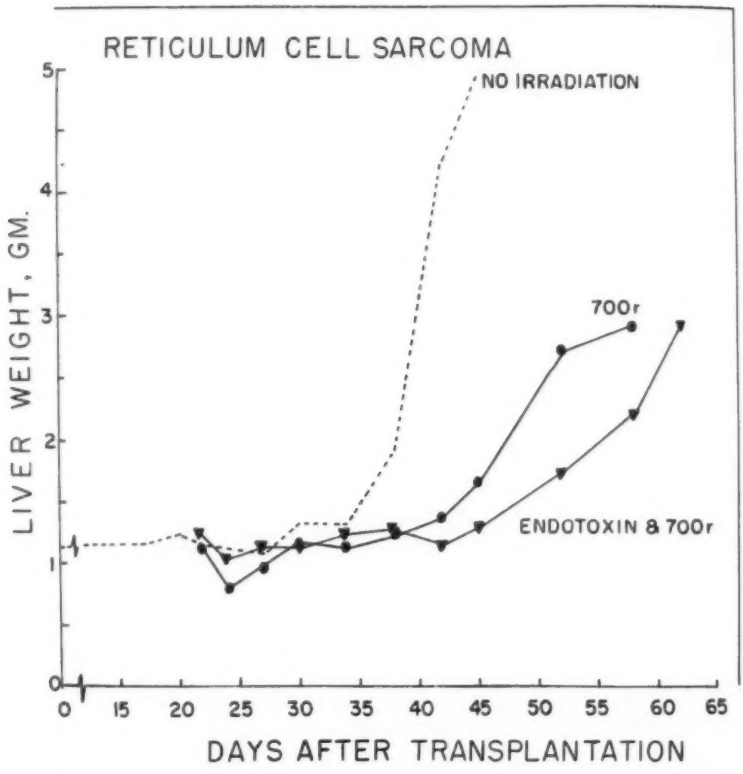
†Three exposures of 500 r each at 9 a.m., 11 a.m., and 1 p.m. followed by a bone-marrow injection. Repeated 36 days later.

‡Three exposures of 500 r each on days 20, 21, 22, the last followed by a marrow injection. Repeated 36 days later.

gained weight very rapidly from the 30th day after transplantation (day 10 on the time scale of the figure) until death some 15 days later. While these mice were gaining 25 percent of their body weight, the gain of control mice amounted to only 7 percent. During this time, the tumor-bearing mice exposed to 600 or 700 r gained about 10 percent of their preirradiation body weight. Tumor-bearing mice exposed to 1000 or 1500 r and injected with bone marrow showed weight changes that did not differ from those in corresponding groups with no tumor.

The increase in liver weight, as shown in text-figure 4, was largely responsible for the terminal increase in body weight of the untreated tumor-bearing mice. The data in this figure are from an experiment in which mice bearing the tumor were treated with endotoxin (day 20), 700 r (day 21), or both, and were killed serially for a study of tumor growth in liver and spleen (expt. 8). Each point represents the mean of 2 or 3 mice. In the untreated animals practically all the increase in liver weight took place during the last 10 days of expected life. Exposure to 700 r delayed the beginning of this weight increase for approximately 7 days and also slowed the rate of gain. In untreated mice gains averaged 3 gm. in a week very near the end of life (days 38 to 45), while livers of irradiated mice gained only 1 gm. in a comparable period a week later (days 45 to 52). During that week the livers of mice treated with endotoxin prior to irradiation gained only 0.5 gm.

No tumor tissue was identified in liver sections made 13 days after transplantation but it was seen in 2 of 3 specimens on day 17. After irradiation it was first identified at 14 days. Three weeks after irradiation (42 days after transplantation) tumor tissue was estimated to be 1 to 10 percent of the liver volume compared with 75 percent or more in un-



TEXT-FIGURE 4.—Liver weights of mice bearing reticulum-cell sarcoma, H6867.

treated mice. The microscopic appearance of the liver in several of these specimens is shown in figures 3, 4, and 5.

Spleen weights of the untreated mice increased steadily from 90 mg. on day 27 to 300 mg. on day 45. In irradiated mice, the study was complicated by the splenic hyperplasia characteristic of recovery from radiation damage (*10*), spleens of irradiated controls reaching a mean weight of 330 mg. 17 days after exposure. In mice with tumors, spleen weights remained low (20 to 25 mg.) for 10 days after irradiation and varied from 90 to 450 mg. during the last few weeks of expected life.

Liver and spleen weights of animals that succumbed to tumor or irradiation are shown in table 6. In untreated mice the liver averaged 4.5 times and the spleen 3 times normal weights. With increasing radiation (and survival time) these organs did not attain such weights. Liver weights in the mice given endotoxin before irradiation were approximately the same at the time of death as in parallel groups given no endotoxin (600 to 700 r, 3.4 gm.; 1100 r, 2.3 gm.; and 500 r × 5, 1.5 gm.), though their survival times were 6 to 11 days longer. Spleen weights at death were also approxi-

mately the same in mice given endotoxin as in those given no endotoxin prior to irradiation.

TABLE 6.—*Liver and spleen weights at the time of death in mice with reticulum-cell sarcoma*

Expt. No.	Dose (*r*)	Number of mice	Liver (*gm.*)	Spleen (*mg.*)	Days survived
4–7	None	61	4. 9	206	45
4	600–700	29	3. 1	162	57
5–6	1000–1100 (M)	31	2. 4	145	63
6	1500 (M)	35	2. 6	122	65
6	1000 × 2 (M)	18	1. 7	70	72
6	1500 × 2 (M)*	16	1. 7	71	72
6	1500 × 2 (M)†	17	2. 3	81	87
7	500 × 5	22	1. 5	93	86
5–6‡	1000–1500 (M)	11	0. 8	39	30

*Three exposures of 500 r each separated by intervals of 2 hours followed by a marrow injection. Repeated 36 days later.

†Three exposures of 500 r each separated by a day, followed by marrow. Repeated 36 days later.

‡Animals from the single exposure experiments that died in less than 3 weeks (mean=10 days) after irradiation.

Discussion

Limitations in the usefulness of whole-body irradiation for cancer therapy stem largely from an insufficient sensitivity differential and a tendency for effectiveness to diminish with increments in dose at the higher radiation levels. Mice can be made to tolerate supralethal radiation by the use of bone-marrow injections, but the resulting therapeutic advantage may be slight. By using an agent which enables the animal to tolerate radiation and which may at the same time act as a deterrent to tumor growth—in the present case a lipopolysaccharide from *S. typhosa*—we hoped to achieve improvement beyond that expected from the sum of the two separate treatment effects.

In experiments with the reticulum-cell sarcoma the combined treatment was superior to the sum of the two single treatments. This does not, however, constitute evidence of sensitization of the tumor cells to X ray. Effects in excess of summation may be caused by: 1) sensitization of the cell by interference with its metabolism, either as a direct effect or through changing its environment; or 2) alterations in the host that would enable it to withstand tumor damage, radiation damage, or both.

Growth of the reticulum-cell sarcoma (as indicated by liver weight) in mice given endotoxin before irradiation lagged behind that of mice not given endotoxin. Endotoxin causes hemorrhage and other vascular changes (*11*), which may well be more pronounced in irradiated than in nonirradiated animals. The resulting tumor necrosis may then be responsible for all of the observed effect. Endotoxin is also known to affect cellular activity (*5*, *12*) and through the same mechanism, at present unknown, it may also influence sensitivity to radiation. The fact that no evidence of sensitization was seen in the lymphosarcoma experiments and the essentially negative results of Lawrence and Duran-Reynals (*13*) make it appear that an increase in sensitivity of tumor cells in general should not

be expected from endotoxin treatment, though a synergistic action, such as was reported for clinical trials by Putnam and his associates (*14*), cannot be ruled out.

Endotoxin treatment did not increase the maximum amount of reticulum-cell sarcoma that could be tolerated, assuming that liver weight and degree of involvement correspond adequately to tumor growth. At death the weight and tumor involvement of the liver were approximately equal in the mice that were treated and those that were not treated with endotoxin.

Endotoxin did, on the other hand, increase the amount of radiation that could be tolerated by the tumor-bearing mice by stimulating an early recovery of hematopoiesis. Tumor-bearing mice treated with marrow or endotoxin survived the acute effects of radiation as well as did mice without tumors. Radiation was a contributory cause of late deaths, however, even in the groups that were most benefited by it. In these cases the liver at the time of death was not a great deal larger than normal and showed much less tumor involvement than did the liver of non-irradiated mice at death. The increase in survival time of the endotoxin-treated mice in these experiments may be specifically attributable to a stimulation of hematopoiesis, though this was not determined.

References

(*1*) Shear, M. J.: Chemical treatment of tumors. IX. Reactions of mice with primary subcutaneous tumors to injection of a hemorrhage-producing bacterial polysaccharide. J. Nat. Cancer Inst. 4: 461–476, 1944.

(*2*) Duran-Reynals, F.: Reaction of transplantable and spontaneous tumors to blood-carried bacterial toxins in animals unsusceptible to the Schwartzman phenomenon. Proc. Soc. Exper. Biol. & Med. 31: 341–344, 1933.

(*3*) Creech, H. S., Hamilton, M. A., and Diller, I. C.: Comparative studies of the immunological, toxic, and tumor-necrotizing properties of polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*). Cancer Res. 8: 318–329, 1948.

(*4*) Smith, W. W., Alderman, I. M., and Gillespie, R. E.: Increased survival in irradiated animals treated with bacterial endotoxins. Am. J. Physiol. 191: 124–130, 1957.

(*5*) ———: Hematopoietic recovery induced by bacterial endotoxin in irradiated mice. Am. J. Physiol. 192: 549–556, 1958.

(*6*) Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G.: Studies on the O antigen of *Salmonella typhosa*. I. Purification of the antigen. J. Immunol. 74: 455–465, 1955.

(*7*) Landy, M., Johnson, A. G., Webster, M. E., and Sagin, J.: Studies on the O antigen of *Salmonella typhosa*. II. Immunological properties of the purified antigen. J. Immunol. 74: 466–478, 1955.

(*8*) Landy, M. and Johnson, A. G.: Studies on the O antigen of *Salmonella typhosa*. IV. Endotoxic properties of the purified antigen. Proc. Soc. Exper. Biol. & Med. 90: 57–62, 1955.

(*9*) Landy, M., and Pillemer, L.: Increased resistance to infection and accompanying alteration in properdin levels following administration of bacterial lipopolysaccharides. J. Exper. Med. 104: 383–409, 1956.

(*10*) Smith, W. W., Anderson, W. Jr., and Ashwell, G.: Spleen adenosine triphosphatase activity in irradiated mice treated with spleen homogenate. Am. J. Physiol. 178: 471–473, 1954.

(11) ALGIRE, G. H., LEGALLAIS, F. Y., and ANDERSON, B. F.: Vascular reactions of the normal and malignant tissues *in vivo*. V. The role of hypotension in the action of a bacterial polysaccharide on tumors. J. Nat. Cancer Inst. 12: 1279–1295, 1952.

(12) WINDLE, W. F., CHAMBERS, W. W., RICKER, W. A., and GINGER, L. G.: Reaction of tissues to administration of a pyrogenic preparation from a pseudomonas species. Am. J. M. Sc. 219: 422–426, 1950.

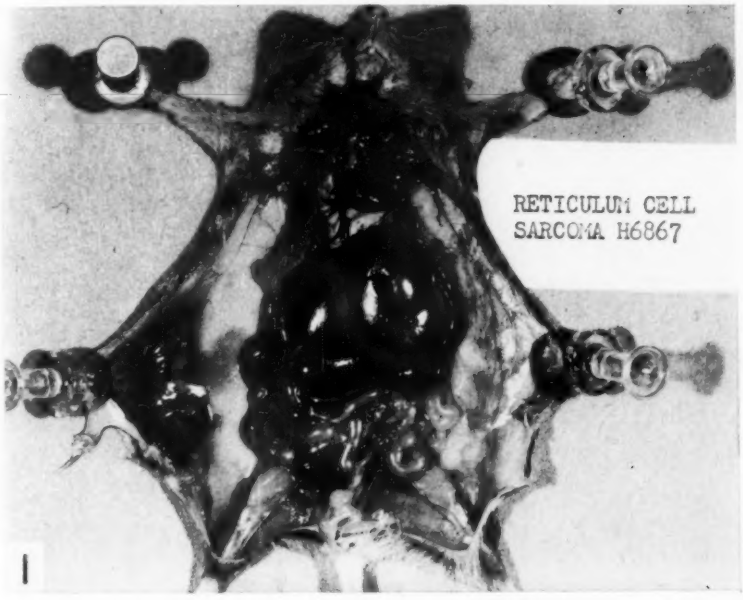
(13) LAWRENCE, E. A., and DURAN-REYNALS, F.: The effect of combining bacterial toxins and X-ray irradiation in the treatment of a transplantable mouse carcinoma. Yale J. Biol. & Med. 14: 177–181, 1941.

(14) PUTNAM, R. C., POMEROY, T. C., DONNELLY, A. J., NISHIMURA, E. T., BOWMAN, H. S., and REIMANN, S. P.: The clinical use of bacterial polysaccharides in malignant disease. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 240, 1957.

PLATE 40

FIGURE 1.—(BALB/c × DBA/2)F_1 female bearing reticulum-cell sarcoma, H6867, which died 31 days after transplantation. *Note* large liver, spleen, and healthy tumor.

FIGURE 2.—(BALB/c × DBA/2)F_1 female bearing reticulum-cell sarcoma, H6867, which died 11 days after 800 r (31 days after transplantation). Liver and spleen are small and the tumor is hemorrhagic.


RETICULUM CELL
SARCOMA H6867
1

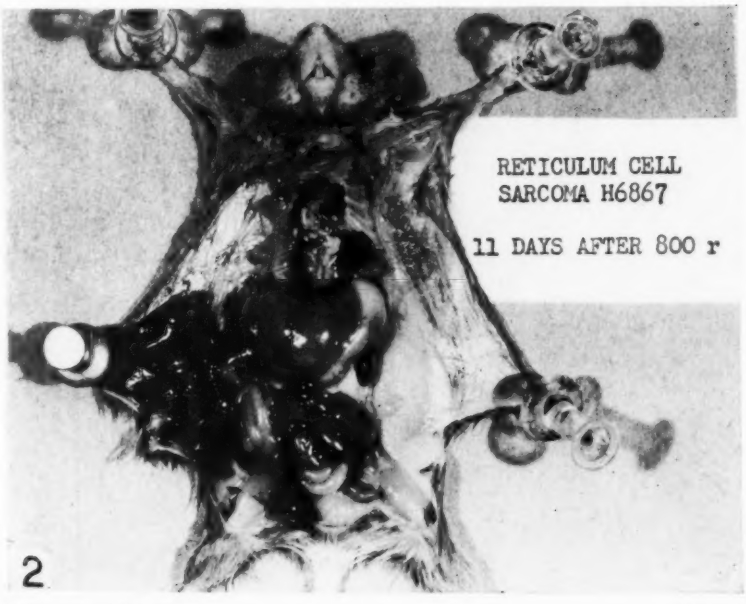
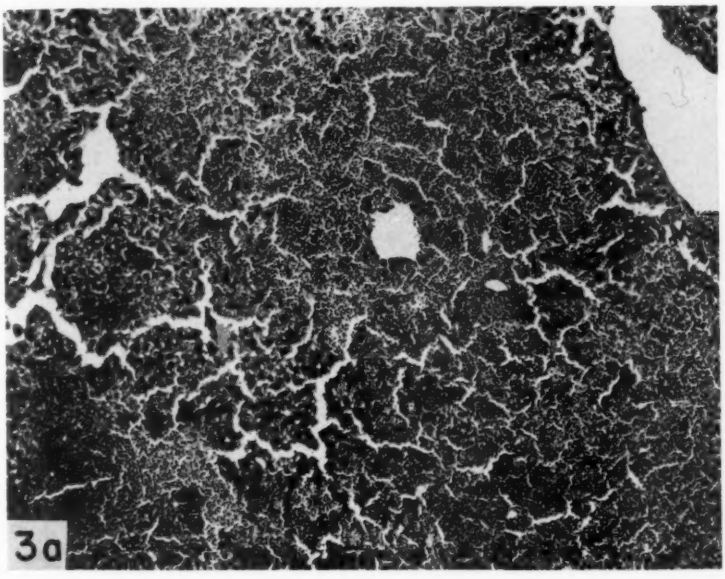

RETICULUM CELL
SARCOMA H6867
11 DAYS AFTER 800 r
2

PLATE 41

FIGURE 3a.—Liver of mouse bearing reticulum-cell sarcoma, H6867, which was killed 45 days after transplantation. × 63

FIGURE 3b.—Higher magnification of same section. × 285


3a

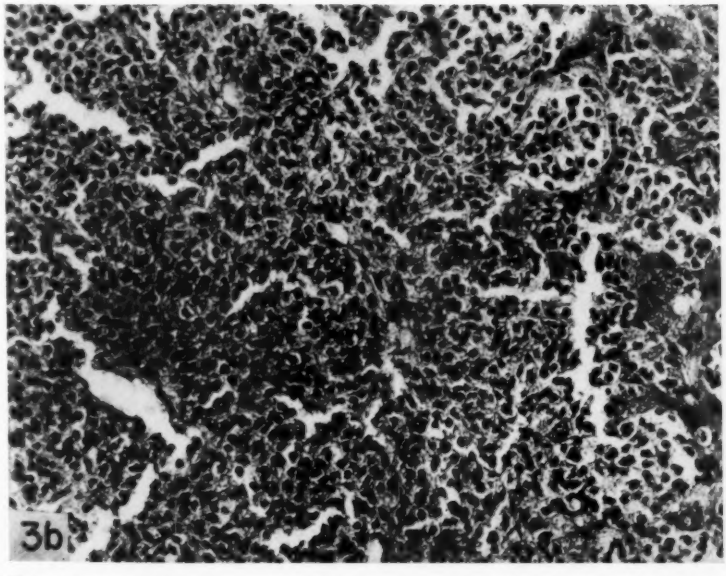

3b

PLATE 42

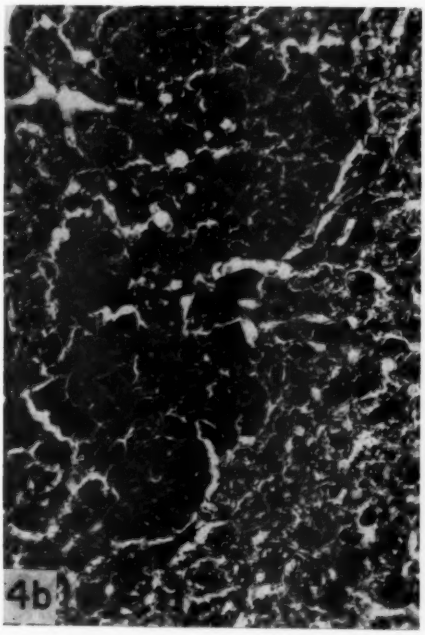
FIGURE 4a.—Liver of mouse bearing reticulum-cell sarcoma, which was irradiated with 700 r on the 20th day and killed on the 45th day after transplantation. × 63

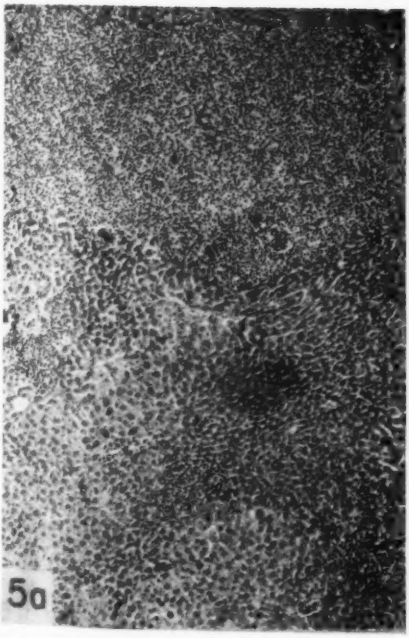
FIGURE 4b.—Higher magnification of same section. × 285

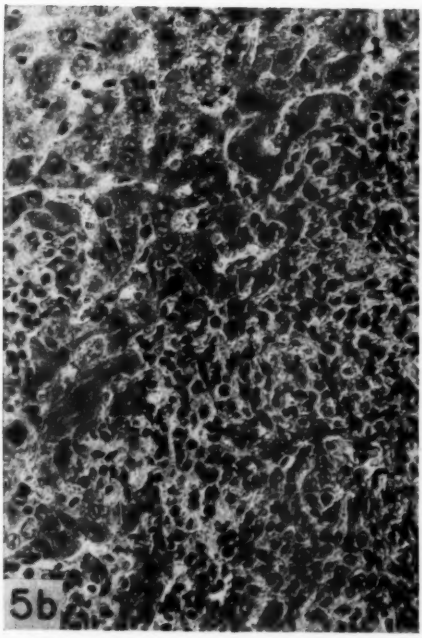
FIGURE 5a.—Liver of mouse bearing reticulum-cell sarcoma, which was irradiated with 700 r on the 20th day and killed when moribund on the 58th day after transplantation. × 63

FIGURE 5b.—Higher magnification of same section. × 285

4a


4b


5a


5b

Massive Fluid-Suspension Cultures of Certain Mammalian Tissue Cells. I. General Characteristics of Growth and Trends of Population [1, 2, 3]

JAY C. BRYANT, EDWARD L. SCHILLING, *and* WILTON R. EARLE, *Tissue Culture Section, Laboratory of Biology, National Cancer Institute,*[4] *Bethesda, Maryland*

Summary

J. Nat. Cancer Inst. 21: 331–348, 1958

Studies on the growth of mammalian cells in fluid-suspension cultures under conditions of continuous aeration and agitation were continued, and additional strains of cells were grown successfully.

The manner of growth in fluid-suspension cultures appeared to be a specific characteristic of the cell strains studied and ranged from single cell suspensions (NCTC clones 929 and 1469) to rather large compact aggregates (NCTC strains 1769 and 1985).

Noncellular debris nearly always formed in fluid-suspension cultures of human skin epithelium (NCTC strain 1769) regardless of culture condition, but seldom formed in cultures of strain L (NCTC clone 929) or in cultures of mouse liver cells (NCTC clone 1469), and then only when necrosis was present. Debris appeared to originate from the serum.

Average generation time was usually 2 days or more in length. Cultures of strain 1769 had the longest average generation time, 5.0 days, while cultures of clone 929 and liver clone 1469 had generation times of 3.6 and 3.2 days, respectively. The shortest generation time shown by any of the cultures reported here was 2.25 days for a culture of skin strain 1769.

Irregular growth curves characterized skin strain 1769 cultures, while smooth growth curves were typical of cultures of clones 929 and 1469.

When subculturing was delayed until the logarithmic phase had ended, the parent culture was often so necrotic that recovery by the daughter culture into a logarithmic phase was slow and uncertain.

[1] Received for publication February 19, 1958.

[2] Much of these data were originally reported at the annual meetings of the Tissue Culture Association at Madison, Wis., on April 6, 1956, and at Baltimore, Md., on April 16, 1957.

[3] Since this manuscript was submitted for publication the following papers pertinent to this general field have come to the attention of the authors:

1) Danes, B. Shannon: Suspension cultures of strain L mouse fibroblasts. I. A glass stirrer apparatus for the cultivation of cell suspensions. Exper. Cell Res. 12: 169–179, 1957.

2) Graff, S., and McCarty, K. S.: Sustained cell culture. Exper. Cell Res.: 13: 348–357, 1957.

3) McLimans, W. F., Davis, E. V., Glover, F. L., and Rake, G. W.: The submerged culture of mammalian cells: the spinner culture. J. Immunol. 79: 428–433, 1957.

4) McLimans, W. F., Giardinello, F. E., Davis, E. V., Kucera, C. J., and Rake, G. W.: Submerged culture of mammalian cells: the five-liter fermentor. J. Bact. 74: 768–774, 1957.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Rapid proliferation of several strains of animal cells in the form of free-floating, rapidly agitated cell suspensions in various kinds of culture flasks has been reported from this laboratory (*1–3*) and by other workers (*4–7*). Owens, Gey, and Gey (*4*) studied the growth of lymphoblastic mouse tumor cells in "tumbling tube" cultures *in vitro*. In this laboratory, suspensions of NCTC clone 929 [5] of strain L mouse fibroblasts grew luxuriantly in specially designed roller tubes operating at relatively high rates of drum rotation (*3*); these cultures showed a well-defined lag phase, lasting 24 hours or less, preceding a clearly expressed logarithmic growth period. In further studies of cell suspensions, three established cell strains proliferated exponentially to high population density in modified flat-bottom, boiling flasks of 1,000 ml. and 1,500 ml. size, agitated rapidly on a New Brunswick type shaker (*2*). Eichorn,[6] McLimans *et al.* (*7*), and Cherry and Hull [7] have used various magnetic stirrer culture systems for maintaining cells of various strains in fluid suspensions in a state of continuous growth for several weeks. Graham and Siminovitch (*5*) reported that, in small test tubes rotated at 40 to 50 r.p.m., monkey kidney cells proliferated well when cells were subcultured during the logarithmic phase of growth.

Rinaldini (*9*), Graham and Siminovitch (*5*), Kuchler and Merchant (*6*), Puck, Marcus, and Cieciura (*10*), and Yamada and Takano (*11*) have reported characteristic growth curves for mammalian tissue cells grown in tissue cultures, either in static substrate or in agitated fluid-suspension cultures like those for microbial populations.

The present paper, first in a series under this general title, reports continuation of studies (*2*) on the growth of cells of various established strains in fluid-suspension culture under conditions of reasonably rapid aeration and continuous agitation. Major emphasis is placed on the proliferation of cells of these strains during the logarithmic phase of growth. Qualitative observations are also reported on characteristic manners of cell growth and on the formation of noncellular protein in suspension. Other phases of the study will be covered in later papers.

Materials and Methods

Cell strains.—Cell types used in this study included the 3 cell clones or strains used in cell suspension studies that have been reported previously (*2*): NCTC clone 929 from mouse fibroblast strain L (*12*), NCTC clone 1469 from mouse epithelial cells of the parenchyma of the liver (*13*), and the HeLa strain from a human squamous-cell cervical carcinoma (*14*,

[5] Since references to cell strains, clones, and lines are becoming increasingly frequent in the tissue culture literature, all cell strains originating in the Tissue Culture Section, Laboratory of Biology, National Cancer Institute, are currently being designated by the prefix "NCTC strain" before the number in order to avoid confusion. Clones will be designated as "NCTC clone" before the number, and cell lines within a clone or a strain will be designated as "NCTC line" before the number.

For a summary of the history of NCTC clone 929 mouse fibroblast *see* footnote in the paper by McQuilkin, Evans, and Earle (*8*).

[6] Dr. Paul A. Eichorn, personal communication.

[7] From a paper presented to the Tissue Culture Association at Milwaukee, Wis., April 3, 1956: Studies on the growth of mammalian cells in agitated fluid media, by W. R. Cherry and R. N. Hull.

15), the cells from one line of which, as grown in this laboratory, are designated strain 1985. Two other cell strains or clones used in this study were *a*) NCTC strain 1769 [8] derived from normal adult human skin epithelium and maintained in tissue culture in this laboratory since November 1953, and *b*) NCTC strains 2071 and 2168 (*8*) which had been adapted from NCTC clone 929 so that they could be maintained in reasonably rapid proliferation in protein-free, chemically defined medium NCTC 109, and in NCTC 109 plus 0.25 percent horse serum.[9]

Handling of cultures.—Cultures from established cell strains in T-60 or T-30 flasks (*17*) were planted in shaker flasks as unsieved suspensions of cells in freshly added fluid after the 48- to 72-hour-old fluid had been withdrawn. Fresh culture fluid was used to dilute the suspension to the desired population density in the culture flask. Nuclei counts were made periodically on small aliquots of the suspension, which will be described. Transfer of cultures and changes of culture fluid were carried out as previously described (*2*).

The cultures from which data are presented were actually selected from a large group of experiments on the basis of showing a well-defined logarithmic growth phase at least several days in length.

Culture media.—Culture media used in various experiments are shown in table 1. Large as well as small molecule components were supplied in serum. Small molecule components were supplied in whole egg ultrafiltrate (*18*) or in a chemically defined medium. In the latter, protein-free, chemically defined medium NCTC 109 [10] was prepared at 2.5 times its final concentration and constituted 40 percent of the complete medium by volume; Earle's saline (*21*) and specified volumes of horse serum, within the range from 5 to 20 percent, constituted the other 60 percent of the complete medium.

Equipment and methods for incubation and agitation.—The shaker, incubator, and culture-flask design used in this study have been described (*2*). The maximum oscillation rate of the shaker, 12,600 revolutions or oscillations per hour, was generally used for all experiments preceding experiment #2260. The speed was reduced to 8640 r.p.h. for all other experiments reported in this paper, *i.e.*, those above 2260. Gassing equipment for a continuous slow flow of gas through each culture above the fluid was the same as previously described (*2*). Two modifications were made in the method of gassing the cultures: 1) Both the outlet gas filter and the inlet filter on each culture flask were changed at every fluid renewal in order to exclude the possibility of bacteria growing through the cotton filter. 2) The rate of gas flow through the flask above the fluid

[8] In the original paper on this strain (*16*), it was stated that the cells were taken from the skin of a 65-year-old man; a later check of the actual clinical records showed the man was 52 years old.

[9] In further reference to these strains in this paper the prefixed letters NCTC will be omitted and the names will also be shortened to the following forms, in the order mentioned: clone 929, liver clone 1469, HeLa strain 1985, skin strain 1769, strain 2071, and strain 2168.

[10] NCTC 109 is the same as NCTC 107 previously described (*19*, *20*) except for 3 modifications: 1) the addition of vitamin B_{12} at a concentration of 0.01 mg. per 100 ml., 2) the omission of unsaturated fatty acids and cholesterol, and 3) a reduction in concentration of Tween 80 to 55 percent of that originally specified. Earle's saline (*21*) was the source of other small molecule components.

was doubled for some cultures when the number of cells became very great or the rate of proliferation was high. This was accomplished by joining the tubes from 2 metering units into 1 flask. The speed of the multiple unit metering device was kept constant at all times. It was thus possible to secure 2 different rates of gas inflow, 140 ml. per hour and 280 ml. per hour.

Enumeration of nuclei.—The cell suspension was sampled for a nuclei count at the time of planting (inoculum count), and at each fluid change or fluid addition throughout the entire life of each culture. The nuclei enumerated were those which showed typical chromatin structure and normal staining (*23*). The method of enumerating the nuclei in a 1 ml. aliquot from each culture flask was essentially the same as that previously described (*2*, *23*) for all nuclei counts made before September, 1955. No experiments reported in this paper were then in progress. After that time the following modification of this method was used for all cultures in this study. A 1 ml. aliquot of the cell suspension was withdrawn from the culture; it was pipetted directly into a mixture of 4 ml. of 0.1 M citric acid and 1 ml. of 0.1 percent crystal violet in 0.1 M citric acid. After standing 5 to 10 minutes at room temperature, the mixture was drawn up into rotor pipettes; [11] after 10 minutes rotation the nuclei were counted in hemocytometer chambers [12] in the usual manner. It was possible to complete a nuclei count within 30 minutes after sampling the suspension, and to use this relatively reliable estimate of population for determining the optimum volume of new fluid to be added at each fluid change. By emphasizing critical steps in this method of preparation of the cells for nuclei enumeration, even the large aggregates of cells of human skin epithelium studied in several experiments were broken up satisfactorily into discrete, countable nuclei. The most important compensatory measure for sampling suspensions containing these aggregates was in taking two 1 ml. cell suspension aliquot samples instead of one. The behavior of the large cell aggregates in HeLa cell-suspension cultures under the conditions of this shortened procedure, however, has not been determined.[13]

Experiments and Results

Characteristic Manner of Growth of Cell Strains

Various cell strains [14] in agitated fluid-suspension cultures showed major differences in their manner of growth. The liver clone 1469 cultures proliferated as suspensions of single cells with only few, small, loose aggregates of cells. The clone 929 cultures proliferated primarily as

[11] The adjustable pipette clamps on the Bryan-Garry blood-counting pipette rotor have been reset, as noted previously (*24*), for use in all nuclei enumerations in this laboratory. The angle between the axis of the pipette and the axis of the rotor shaft was increased from 12° to 22° in order to insure more thorough agitation.

[12] American Optical Company, Spencer Bright Line Improved Neubauer ruling.

[13] This shortened procedure for the nuclei enumeration method has not been applied to cultures in any of the stationary flasks (T-9, T-15, T-30, T-60, and D-3.5 flasks) used in other studies in this laboratory. In such stationary flasks, cells tend to proliferate on the floor of the flask in more or less closely adhering sheets. These sheets yield rather slowly to citric acid treatment, requiring considerable incubation time at 37° C. Hence, this shortened procedure may be unsuitable for static surface substrate cultures.

[14] *See* footnote 9.

suspensions of isolated cells and also as loose, amorphous aggregates of cells. The HeLa strain 1985 cultures and the skin strain 1769 cultures proliferated so that the suspensions consisted primarily of numerous rounded or spheroidal, tightly bound aggregates of cells.

The liver clone 1469 cultures tended to form a sheet of cells on the floor and lower sides of the culture flask. The cell sheets were generally not wholly continuous and tended to be somewhat irregular and unstable. The presence of such a sheet of cells on the floor of a shaker culture flask obviously introduced an indeterminate degree of error into the nuclei counts because the 1 ml. aliquot of suspension taken for the count was not truly representative. Such a sheet of cells formed in culture 2163-1-1 of liver clone 1469 (text-fig. 1) and persisted during the latter part of the long lag phase and the first half of the logarithmic phase, *i.e.*, from the 7th to the 23rd day. The smoothness of the nuclei population curves for this culture and for the other mouse liver cultures suggests, however, that the sampling error from this cause was relatively constant and probably insignificant.

In cultures of HeLa strain 1985 and in cultures of skin strain 1769, the aggregates of cells gradually increased in size, over several days, some reaching 1 millimeter or more in diameter. The center of some of these larger spheres, in some instances, became more opaque and apparently necrotic. These 2 strains usually have relatively few isolated cells in suspension. Attempts to either break down the aggregates or inhibit the formation of aggregates in cultures of skin strain 1769 were relatively ineffective. It appeared that increasing the velocity of the shaker to 12,600 r.p.h. had only a limited effect in dispersing the aggregates or inhibiting their formation. Further study of the nature and control of this phenomenon is needed for more precise quantitative studies of such strains of cells in agitated suspension cultures.

Cell Proliferation During the Logarithmic Phase of Growth

A logarithmic phase characterized at least some parts of the population curves of the agitated suspension cultures reported in this study. An expression for generation time [15] has been applied to the relatively rapid phase of growth, presumably logarithmic, of some of the agitated cell-suspension cultures that have been carried in this laboratory. Table 1

[15] Formulas developed for average growth rate and generation time in bacterial cultures (*25–27*) have been used in this study. Briefly, the population n_2 after m generations is equivalent to 2^m times the number of nuclei in the original inoculum n_1, or

$$n_2 = n_1 2^m$$

This equation may be expressed logarithmically as

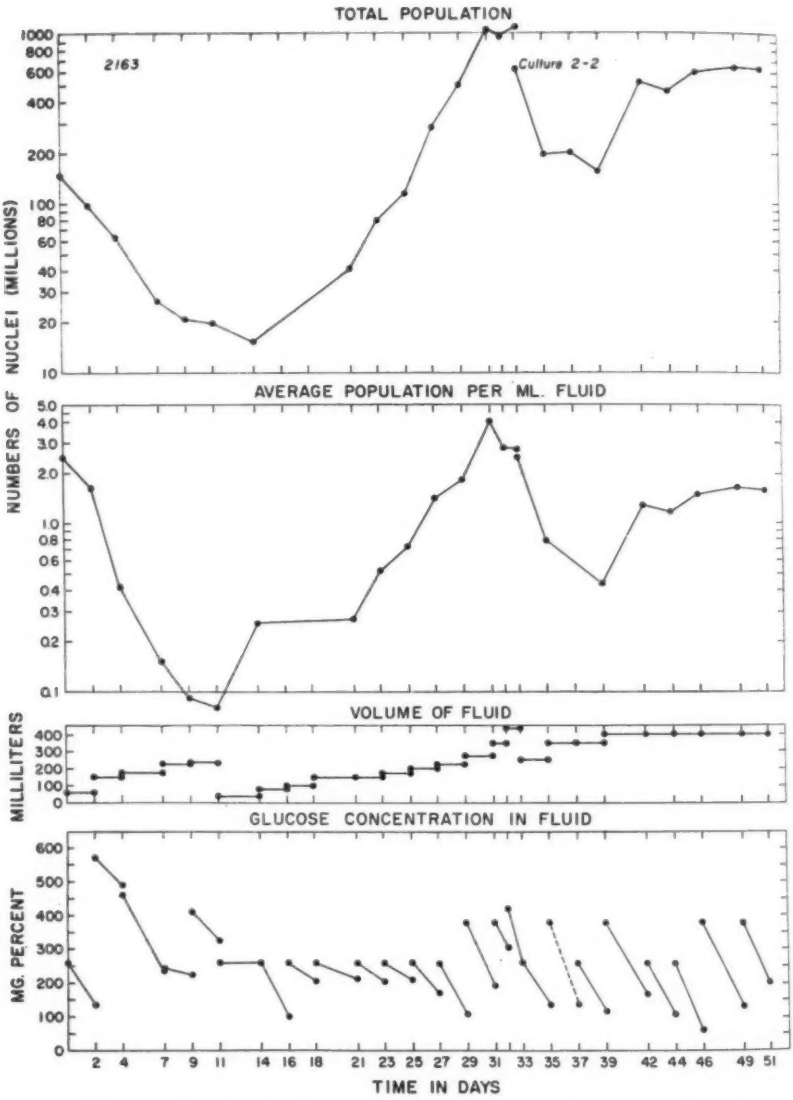
$$\log n_2 = \log n_1 + m \log 2$$

$$m = \frac{\log n_2 - \log n_1}{\log 2} = 3.32(\log n_2 - \log n_1)$$

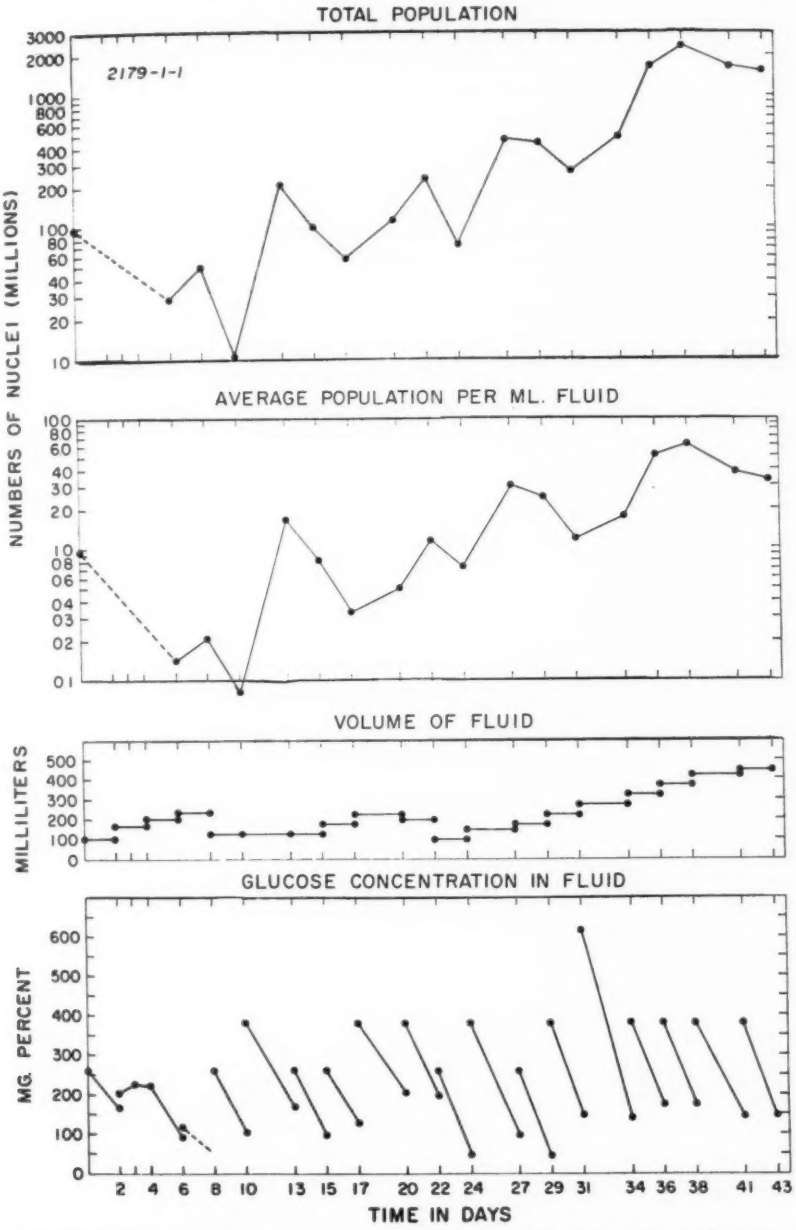
From this the generation time g may be calculated:

$$g = \frac{t_2 - t_1}{m}$$

The term "generation time" in this context is used to indicate the average life span of the organism, which in the case of individual bacteria and metozoan cells is terminated by either mitosis or necrosis. This term must be clearly distinguished, however, from the term "transfer generation" as used in this laboratory to indicate the period of maintenance of a culture as one entity in one culture flask or other container.



TEXT-FIGURE 1.—Cell proliferation curve and other data characterizing fluid-suspension culture 2163-1-1 and daughter culture 2163-2-1 of liver NCTC clone 1469 cells. Each point on the curves represents a complete fluid change except the fluid additions on the 2nd, 4th, and 7th days. Parent culture was subcultured on the 33rd day of the experiment. Cell proliferation values are actually derived from numbers of nuclei counted in aliquot samples.



TEXT-FIGURE 2.—Cell proliferation curve and other data characterizing fluid-suspension culture 2179-1-1 of human skin NCTC strain 1769 cells. Each point on the curves represents a complete fluid change except the fluid additions on the 2nd, 4th, and 6th days. Cell proliferation values are actually derived from numbers of nuclei counted in aliquot samples.

shows the proliferation levels and the generation time of representative suspension cultures of various cell strains during the logarithmic phase of growth.[16] The cultures are listed, first, in groups according to the cell strain, and, second, within each group in the chronological order in which the experiments were carried out.

Average generation time, in table 1, differs consistently between the various cell strains under the experimental conditions used. The cultures of skin strain 1769 showed the longest average generation time, as a group, during their entire logarithmic phase, 5.04 days, and the clone 929 cells on NCTC 109 medium supplemented with horse serum had the shortest, 2.68 days. In the latter case, particularly, the data appear to be quite characteristic of the specific cell strain on the specific medium. HeLa strain 1985 cells in experiment 1939 proliferated in the logarithmic phase with somewhat shorter generation times, 2.66 and 3.04 days, respectively, than most of the cultures of skin strain 1769 cells, despite the close similarity in manner of growth of these 2 strains and in the media used for these cultures. The 7 cultures of clone 929 cells on 20 percent whole egg ultrafiltrate (WUF) and 40 percent horse serum had generation times that averaged 3.16 days.

Smooth logarithmic growth curves, reflecting uniform rates of increase, generally characterized cultures of clone 929 cells and cultures of liver clone 1469 cells (text-fig. 1). Although a less uniform curve had been shown by culture 1740 of the latter clone, (*2*), a smooth curve has most often typified this liver clone in other cell-suspension cultures not reported here. A smooth type of curve during the logarithmic phase is based upon 1) a uniform rate of cell proliferation, 2) a cell suspension comprised largely of isolated cells or, at most, only small, loose, amorphous aggregates, permitting the relatively small aliquot samples taken for nuclei counts to be more completely representative, and 3) the absence of severe necrosis. Agitated fluid-suspension cultures of these two cell clones are therefore highly satisfactory for quantitative population studies.

Irregular population curves, even in the logarithmic phase of growth, generally characterized skin strain 1769 cells in agitated fluid suspension. This characteristic undoubtedly resulted from the tendency of these cells to proliferate in the form of compact rounded aggregates, with relatively few isolated cells in suspension. The aggregates settled out very rapidly as soon as the culture flask was taken off the shaker platform for fluid change. Consequently, truly representative aliquot samples of the cell suspension for nuclei counts were exceedingly difficult to obtain, while the tendency for the center of the larger aggregates of cells to become necrotic was often appreciable. The record of culture 2179-1-1 may be cited (text-fig. 1, and table 1). This culture appeared to pass into the logarithmic growth phase on the 10th day, and to proliferate constantly in that phase for 28 days, despite considerable fluctuations of the growth

[16] Only 2 cultures out of many carried on NCTC 109 medium are shown here; most of the experiments with that medium will be reported in a separate paper. Included in table 1 are 2 cultures for which the graphs of population are shown in text-figures 1 and 2, culture 2163-1-1 of liver clone 1469 and culture 2179-1-1 of skin strain 1769.

TABLE 1.—*Proliferation levels and generation time of representative suspension cultures of various cell strains during the logarithmic phase of growth*

Cell strain	Culture designation†	Components of culture medium* (*v/v*)				Logarithmic phase in life of culture (*day*)			Nuclei numbers (*millions*)			Generation time *g* (*days*)	Average generation time of cell strains (*days*) §
		Small molecule		Serum									
		Percent	Kind‡	Percent	Kind	t_1	t_2	t_2-t_1	n_1 At t_1	n_2 At t_2	n_2-n_1		
Human skin epithelium (NCTC 1769)	2179–1–1	20	WUF	40	Human	31	38	7	268. 0	2320. 0	2052. 0	2. 25	5. 04
	2179–1–1	20	WUF	40	Human	10	38	28	10. 3	2320. 0	2309. 7	3. 58	
	2199–1–1	20	WUF	40	Human	21	35	14	189. 0	1971. 6	1782. 6	4. 14	(4. 02)
	2199–1–1	20	WUF	40	Human	0	35	35	28. 0	1971. 6	1943. 6	5. 71	
	2303–1–1	20	WUF	40	Human	0	16	16	32. 1	153. 0	120. 9	7. 12	
	2320–1–2	20	WUF	40	Human	0	5	5	67. 6	257. 6	190. 0	2. 59	
	2320–1–2	20	WUF	40	Human	7	14	7	60. 5	237. 5	177. 0	3. 74	
HeLa (strain 1985)	1939–1–2‖	20	WUF	30	Human	0	19	19	10. 9	1540. 0	1529. 1	2. 66	2. 85
	1939–1–2‖	20	WUF	30	Human	0	24	24	10. 9	2605. 0	2594. 1	3. 04	
Mouse liver (NCTC clone 1469)	1740–1–4‖	20	CEE	30	Horse	13	24	11	15. 9	191. 7	175. 8	3. 06	3. 63
	2163–1–1	20	WUF	40	Horse	14	31	17	10. 3	1103. 0	1092. 7	2. 52	
	2213–1–1	20	WUF	40	Horse	0	9	9	156. 0	1200. 0	1044. 0	3. 06	
	2213–2–1	20	WUF	40	Horse	10	17	7	320. 0	1234. 5	914. 5	3. 60	
	2213–3–1	20	WUF	40	Horse	4	9	5	534. 0	958. 6	424. 5	5. 93	
Mouse strain L (NCTC clone 929)	1912–1–1‖	20	CEE	30	Horse	0	12	12	24. 5	753. 0	728. 5	2. 43	3. 16
	1912–2–3‖	20	CEE	30	Horse	0	4	4	295. 0	779. 0	484. 0	2. 86	
	2068–1–2	20	WUF	40	Horse	2	7	5	44. 7	322. 9	278. 2	1. 75	
	2068–1–2	20	WUF	40	Horse	16	25	9	246. 7	1026. 4	779. 7	4. 38	
	2088–1–1	20	WUF	40	Horse	5	17	12	151. 6	2273. 8	2122. 2	3. 07	
	2090–1–2	20	WUF	40	Horse	2	30	28	15. 3	1460. 3	1445. 0	4. 26	
	2313–1–1	20	WUF	40	Horse	2	7	5	127. 3	427. 0	299. 7	2. 87	
	2313–1–2	20	WUF	40	Horse	2	7	5	133. 7	349. 0	215. 3	3. 62	
Mouse strain L (NCTC clone 929, line 2168)	2260–2–2	40	NCTC 109	20	Horse	0	7	7	60. 9	461. 9	401. 0	2. 40	2. 68
	2260–2–4	40	NCTC 109	20	Horse	0	7	7	96. 2	496. 2	400. 0	2. 96	

*In addition to Earle's saline (*21*).

†In this column the first number identifies the experiment, the second number identifies the transfer generation in that experiment, and the third number identifies the specific culture in that generation.

‡WUF = whole egg ultrafiltrate (*18*); CEE = filtered chick-embryo extract (*22*); NCTC 109 = protein-free, chemically defined medium (*19, 20*), *see* footnote 10.

§5.04 is the average for the "longer" logarithmic growth phase of the NCTC 1769 cultures listed here, while 4.02 is the average for the "shorter" logarithmic growth phase of these cultures; *see* text-figure 2 for the growth curve of one of these cultures.

‖Proliferation curves of these cultures have been presented in text-figures previously (*2*); basic and derived data are included here for comparison.

curve during that interval. The long-term trend, defined in table 1, line 2, only by the nuclei populations at the beginning and at the end of the logarithmic phase, represented an average generation time of 3.6 days. The last 7 days of this phase appeared to be enough steeper and smoother to justify a separate calculation of generation time, which is given in the first line of table 1 as 2.25 days. Similar irregularity was evident in the logarithmic proliferation curves of the other skin strain 1769 cultures, particularly culture 2199-1-1. The data for this culture also show a long over-all logarithmic phase, which held for 35 days during which the average generation time was 5.7 days. A shorter, steeper, smoother part of the logarithmic curve, the last 14 days, was represented by an average generation time of 4.1 days.

Cells of HeLa strain 1985 behaved much like those of the skin strain 1769. The relatively smooth, logarithmic growth curve of HeLa culture 1939-1-2 (*2*), however, was associated with smaller cell aggregates, which allowed more representative aliquot samples for nuclei counts. Whether proliferation in this culture was actually more uniform and constant than that in later cultures of the same strain was not clear from available data.

Maximum population levels reached by certain cultures, listed in table 1, approached but did not equal the maximum of 5.9 billion nuclei reported previously by this laboratory for culture 1939–2–3 of HeLa strain 1985 (*2*). Two cultures of human skin, 2179-1-1 and 2199-1-1, reached the maxima of 2.32 and 1.97 billion nuclei, respectively, which were comparable to the 2.6 billion maximum of culture 1939-1-2 of HeLa strain 1985, parent to the HeLa culture mentioned. One clone 929 culture 2088-1-1, also attained a maximum population, 2.37 billion, well within this range. Population maxima between 1 and 1.5 billion were reached by 2 other clone 929 cultures and 3 liver clone 1469 cultures listed in table 1. All these cultures with population maxima greater than 1 billion received culture media composed of 20 percent whole egg ultrafiltrate (*18*) and 40 percent serum. Under these experimental conditions a maximum nuclei population of more than 1 billion, in about 400 ml. fluid in a 1½ liter culture flask, may be reached at the end of the logarithmic phase by the established cell strains listed in table 1.

Cultures of line 2168, adapted from clone 929, were grown on chemically defined medium NCTC 109 supplemented with horse serum (*8*). Maximum populations of the 2 cultures listed in table 1 were 462 and 496 million; these maxima were somewhat lower than those reached by other cultures of this cell strain and of other cell strains, on the same medium, reported in another paper.

Formation of Noncellular Protein in Suspension

Many of the agitated fluid-suspension cultures tended to form masses of a noncellular precipitate which were kept in suspension with the cells during agitation. Most of this material was carried down with the cells during slow-speed centrifugation at fluid change or subculture so that it

could not be separated from the cells. However, the supernatant from centrifugation usually remained somewhat turbid; it appeared that higher centrifugation speeds than can safely be used without injury to the cells would be required to clear the supernatant.

Conditions that led to the formation of noncellular material in agitated fluid-suspension cultures were studied in several experiments. In 1 experiment, 3 cultures, 2244-1-1, 2245-1-1, and 2246-1-1 each from a different cell strain, *i.e.*, clone 929, liver clone 1469, and strain 2168, respectively, were planted with a medium of NCTC 109 supplemented with 5 percent horse serum and with sufficient high-glucose saline to increase the glucose concentration to 260 mg. percent. During the first 2 days considerable amounts of amorphous debris appeared in all 3 cultures simultaneously, with a sharp drop in nuclei population. On the 3rd day an attempt was made to reduce debris formation and to halt the precipitous population decline by increasing the proportion of horse serum from 5 to 20 percent. At 7 days much debris was still present but in macroscopic appearance it was undergoing a change from a finely amorphous mass to a more discrete distribution in small shapeless aggregates. On the 9th day some debris persisted in all cultures; the population of the liver clone 1469 culture 2245-1-1 had fallen so low that the culture was discarded, but the other 2 cultures were showing significant recovery in both population and appearance. Both cultures remained in the logarithmic phase, 2244-1-1 for 11 days and 2246-1-1 for 17 days. The debris disappeared from both cultures early in the logarithmic phase, apparently because 1) further formation of debris ceased, and 2) the residual debris thrown down with the cells by centrifugation at each fluid change was gradually diluted out to such a degree that it could no longer be recognized in the suspension. It appeared that, in agitated fluid-suspension cultures of clone 929 or derived strain 2168 cells, debris tended to form only when the culture was not proliferating rapidly.

In the meantime, on the 2nd day of this experiment, 2 control flasks were set up without cells, each flask containing 50 ml. medium. The medium in one flask was then being used in the cultures, and in the other flask the medium was a mixture of 5 percent horse serum and 95 percent saline. During the next 2 days debris appeared in both control flasks and this debris was quite similar in appearance to that forming simultaneously in the culture flasks containing cells. The control flasks were carried without fluid change for 5 days, during which time the debris persisted; the preparations were then discarded. At this time 2 other control flasks were set up, one with horse serum and the other with chemically defined medium NCTC 109. During 9 days without fluid change, considerable debris accumulated in the first control flask, while none accumulated in the second. It appeared that the formation of debris in agitated fluid-suspension cultures was not necessarily associated with the presence of cells but did appear to be associated with the presence of serum.

A second experiment was carried out with human skin strain 1769

because debris often formed in fluid-suspension cultures of this strain to such an extent that the suspension was distinctly milky in appearance; this debris interfered seriously with subsequent chemical and physical determinations with the cells. The consistently observed tendency of these cells to proliferate in suspension in the form of large, compact, rounded aggregates suggested the possibility that at least part of the debris could be separated from the cells at each fluid change by allowing the cell aggregates to settle out slowly without centrifugation. The following procedure was used: The entire fluid suspension was pipetted into 100 ml. or 200 ml. centrifuge tubes or into a cylinder and was allowed to stand for about 15 minutes. Then the supernatant was pipetted off and discarded. The cell aggregates in the bottom of the tubes were then replaced in the culture flask with fresh fluid. This procedure usually removed some, but not all, of the debris. Additional debris always appeared in the culture, however, before the next fluid change. This procedure was used regularly for culture 2179-1-1 from the 22nd to the 43rd day and for culture 2199-1-1 from the 14th to the 35th day.

The settling-out procedure for separating debris from the culture presumably involved the loss of at least some of the single cells in suspension, and an attempt was made to determine the amount of possible loss. On the 34th day of culture 2179-1-1, nuclei counts were made on the entire supernatant and also on the usual 1 ml. aliquot from the cell suspension. There were only 880,000 nuclei in the 260 ml. of supernatant, while the entire 275 ml. of cell suspension had 493 million nuclei. Thus less than 0.2 percent of the nuclei were discarded in the supernatant from settling. The result of this observation was like that of other periodic checks. Such nuclei counts suggested that practically all of the cells in human skin suspensions existed as aggregates and that this settling-out method for removing most of this comparatively lightweight debris involved only negligible loss of cells.

Discussion

The characteristic manner of growth in fluid-suspension cultures determined the suitability of a given cell strain for quantitative population studies. Cells of clone 929 and liver clone 1469 proliferated mostly as single cell suspensions, and only to a slight extent as very small, loose aggregates of cells; these cells were well suited for quantitative studies. A major consideration in determining suitability was the degree to which the aliquot sample for nuclei count in this study represented an accurate cross section of the total population; the aliquot sample was often only 0.25 percent of the total volume of the suspension. Cells of skin strain 1769 and of HeLa strain 1985 in these cultures were less satisfactory for quantitative studies because of their tendency to form relatively large aggregates of closely coherent cells, with the resultant difficulty of securing a truly representative aliquot sample for the nuclei count.

The formation of noncellular protein precipitate in agitated fluid-suspension cultures is apparently a fairly constant characteristic of certain

cell strains, though it may occur in cultures of any of the strains studied here if culture conditions, *e.g.*, the nutrient media, become unfavorable for normal proliferation. It is more apt to occur in the lag phase, the stationary phase, or the phase of decline than in the logarithmic phase even with clone 929 and liver clone 1469 cells, insofar as other factors are equal. Precipitate was almost always present in gross amounts in cultures of human skin strain 1769. Perry *et al.* (*16*) had observed extensive occurrence of such debris in the early stages of adaptation of this strain to tissue culture in static T-flasks (*17*); the appearance of debris was associated with a period of poor growth or actual deterioration of the culture. These observations have been supported by the present study with the same strain in agitated fluid-suspension cultures in which debris formation was usually independent of the rate of proliferation. Cultures 2179-1-1 and 2199-1-1 of skin strain 1769 both carried relatively large amounts of noncellular debris at all stages even during the most rapid parts of the logarithmic growth phase.

No precipitate formed, however, in comparably dense cultures of clone 929 and liver clone 1469 except when conditions were unfavorable for normal growth. Precipitate often formed in cell-free control flasks containing the complete culture medium in comparable volume, apparently from the serum. Analysis of some associated factors has been carried out in this laboratory by Westfall *et al.* (*28–30*) who have found the precipitate to be primarily protein in nature; the data to date suggest that extra protein precipitate tends to form in any cell-suspension culture which is agitated continuously and has serum in the medium, and which is at a low level of metabolic activity. The conditions in an actively proliferating, "healthy" culture, however, seem to inhibit the formation of extracellular precipitate. The factors involved have not as yet been defined.

When such considerable and variable amounts of precipitate occur, methods of estimating culture growth based on measurement of the packed volume of centrifuged cells could be subject to gross errors.

The 4 major phases characteristic of bacterial cultures may be recognized in the proliferation curves of the animal tissue cell-suspension cultures grown in this laboratory: 1) the initial lag phase, 2) the logarithmic growth phase, 3) the stationary phase, and 4) the phase of decline.

The lag phase, the logarithmic phase, and the beginning of the stationary phase had been expressed clearly in the proliferation of NCTC clone 929 cells in roller-tube, fluid-suspension culture grown only 4 or 5 days, reported previously (*3*), *e.g.*, series 1608 and series 1649. Here the nuclei count and the subsequent 8-hour nuclei counts of the cell suspension allowed recognition of the lag phase which occurred about 24 hours after planting. The proliferation curves then indicated a logarithmic phase for about 48 hours before gradually sloping off into a stationary phase.

In the first studies with large, agitated suspension cultures from this laboratory (*1*), however, and in the currently reported studies with such

cultures, a lag phase of less than 48 hours' duration could not ordinarily have been detected, because the first nuclei count after planting was not made customarily until 48 or 72 hours later, usually at the first fluid change. An unmistakable lag phase was frequently found in these cultures, however, persisting longer than 48 or 72 hours.

The concept of a growth cycle that involves a regular sequence of phases, long recognized to be useful in characterizing the growth of microorganisms, appears to offer some clarification of the behavior of mammalian cells in tissue cultures. Proliferation in the logarithmic phase obviously represents that part of the life of a tissue culture which is of primary practical importance, and which consequently has received major emphasis in this study. The nuclei count method on which the proliferation curves were based includes only those nuclei that appear normal in morphology and staining reaction and consequently are considered viable. Since the usual formulas for generation time and average growth rate in bacterial cultures (*25*) are based on the assumption that all of the cells counted are viable, the application of these formulas to nuclei counts in the logarithmic phase of growth of mammalian cells in fluid-suspension cultures was justified.

Comparisons between cell strains, as well as between other pertinent variables, were based on a calculated value for generation time during the logarithmic phase. In that phase cells of most strains appeared to divide at an approximately constant rate. Therefore it was assumed that the average generation time during the entire logarithmic phase can be calculated from only two counts, one at the beginning and one at the end of the period of rapid proliferation. The extent to which calculations based upon this assumption agreed with those based on nuclei counts during specific intervening shorter intervals depended on the constancy of the proliferation rate.

Although in table 1 the range within groups was great enough to make the between-group differences insignificant on a strict statistical basis, other experiments not reported here support the concept that, when compared under these experimental conditions and in these media, specific animal cell strains in agitated cell-suspension cultures have characteristic growth rates.

Average generation times in table 1 are all longer than the minimum measured in an earlier study (*3*) of the growth of clone 929 cells in rapidly rotated roller-tube cultures. For example, in experiment 1608 in which culture tubes containing 10 ml. fluid suspension were rotated at 2400 r.p.h., a 24-hour lag was followed by a 2-day logarithmic growth phase; the 1st day was characterized by a 15.8 hour (0.66 day) generation time, and the entire 2-day logarithmic phase had a 25.4 hour (1.06 day) average generation time. Again, in experiment 1649, in tubes rotated at 2400 r.p.h., an 18-hour lag phase was followed by a 56-hour logarithmic growth phase which in the first 32 hours was characterized by a 21.9 hour (0.91 day) generation time; the entire logarithmic phase had a 33.1 hour (1.38 day) generation time. In most of the roller-tube experiments, at

rotation speeds of 300 to 1200 r.p.h., the average generation times during the first 7 days after planting were 46.7 to 51.4 hours (1.95 to 2.14 days). It is concluded that the average generation time for mammalian cells in fluid-suspension cultures has been less than 24 hours only under particularly favorable conditions, and then only for very limited periods of time. Such highly favorable conditions seem to have characterized clone 929 cells in the roller-tube cultures studied earlier (*3*) more often than the same strain and other strains of cells in the agitated fluid-suspension cultures reported in this paper.

Yamada and Takano (*11*) analyzed the growth curves of HeLa strain cells in replicate static cultures containing either 2 ml. or 8 ml. culture media; they distinguished 3 phases, lag, logarithmic, and stationary, and found that the growth curves were reproducible. The growth rate in the logarithmic phase was almost constant among various experiments, with a mean generation time of 36.5 hours. These generation times with static cultures of HeLa cells were somewhat less than those found for fluid-suspension cultures in the present study (table 1) regardless of cell strain.

While the concept of an average generation time during the logarithmic phase has proved to be a useful basis for comparing different fluid-suspension cultures, certain limitations to its application should be considered.

1) As used by bacteriologists the expression is based on the assumption that during the logarithmic phase all the organisms are alive and actively dividing. It seems probable that even under optimum conditions some of the organisms fail to survive. If 30 percent of the organisms produced during a given generation or sequent generations failed to survive, the remaining 70 percent, living organisms, would increase at a more rapid rate. In others words, for a given population increase during a given time, the modified formula [17] would have given a shorter generation time than the formula actually used in this study.[18] This consideration apparently does not apply to our study, however, because the nuclei actually counted represented only normal cells (*23*).

2) The duration of the logarithmic phase was not always clearly defined. The nuclei counts at the beginning and end of the phase may occasionally have been subject to considerable sampling error inherent in taking a small aliquot from large volumes of cell suspensions, particularly for those cell strains which tended to form aggregates.

3) The assumption that multiplication during the logarithmic phase is necessarily regular and maximal may not be strictly accurate, either for bacteria or for suspensions of animal tissue cells. The irregularities in the proliferation curves of some of the cell-suspension cultures reported previously (*2*) and in this paper may reflect the sort of intermittent

[17] Then the formula for growth rate (*25*) would be based on the assumption that the population n_2 after m generations is equivalent to 1.4^m times (instead of 2^m times) the number of nuclei in the original inoculum n_1, or

$$n_2 = n_1 \, 1.4^m, \text{ and } \log n_2 = \log n_1 + m \log 1.4$$

Since $\log 1.4 = 0.1461$, and $1/0.1461 = 6.85$, generation time would be $g = \dfrac{t_2 - t_1}{6.85 \, (\log n_2 - \log n)}$. Calculations for generation time in this paper, however, are all based on log 2.

[18] *See* footnote 15.

growth known to be occasionally exhibited by bacteria (*26*) and apparently also characteristic of animal tissue cells rather than the sampling error; or both factors may be operative in certain instances.

4) Certain nuclei counts separated by a 1-day or 2-day interval have given a steeper proliferation curve than nuclei counts separated by an interval of several days. Most of the logarithmic phases listed in table 1 spanned several intervening nuclei counts which were not used in the calculation of generation times for the entire phase. Usually a particularized curve constructed upon the intervening counts showed only minor deviations from a straight line.

Kuchler and Merchant [19] report that the lag phase in mammalian cells directly reflected a temporary stationary or decreasing level in the DNA content of the cells and is not a function of the simultaneous continuous increase in RNA content which correlated with an increase in cytoplasm mass. They report that mammalian cells behave similarly to bacteria. According to this finding, the nuclei counts in the present study during the lag phase would indicate only the standstill or decline in cell numbers but not the simultaneous increase in cytoplasmic mass. Further study of cell volume-weight relationships at various stages in the growth cycle of fluid-suspension cultures is needed to clarify this point.

With all cell strains, once the population curve had passed the logarithmic phase and had tended to plateau or drop, injury to the culture was already so severe that it was difficult or impossible to rapidly reestablish a new logarithmic phase on replanting the culture. At best, a prolonged lag phase marked the early days of the daughter cultures.

When a suspension culture was in the stationary phase, microscopic examination of the cell nuclei during the nuclei count procedure showed a progressive disintegration which often became extremely rapid and severe in the phase of decline. Pyknosis, disappearance of the chromatin net, diffuse staining, and, ultimately, complete and progressive general disintegration and lysis of the nucleus are characteristic manifestations of the process.

References

(*1*) EARLE, W. R., BRYANT, J. C., and SCHILLING, E. L.: Certain factors limiting the size of the tissue culture and the development of massive cultures. Ann. New York Acad. Sc. 58: 1000–1011, 1954.

(*2*) EARLE, W. R., BRYANT, J. C., SCHILLING, E. L., and EVANS, V. J.: Growth of cell suspensions in tissue culture. Ann. New York Acad. Sc. 63: 666–680, 1956.

(*3*) EARLE, W. R., SCHILLING, E. L., BRYANT, J. C., and EVANS, V. J.: The growth of pure strain L cells in fluid-suspension cultures. J. Nat. Cancer Inst. 14: 1159–1171, 1954.

(*4*) OWENS, O. VON H., GEY, G. O., and GEY, M. K.: A new method for the cultivation of mammalian cells suspended in agitated fluid medium. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 41, 1953.

[19] From a paper presented to the Tissue Culture Association at Baltimore, Md., April 17, 1957: The relationship of protein and nucleic acid synthesis to the development of a mammalian cell population *in vitro*, by R. J. Kuchler and D. J. Merchant.

(5) GRAHAM, A. F., and SIMINOVITCH, L.: Proliferation of monkey kidney cells in rotating cultures. Proc. Soc. Exper. Biol. & Med. 89: 326–327, 1955.

(6) KUCHLER, R. J., and MERCHANT, D. J.: Propagation of strain L (Earle) cells in agitated fluid suspension cultures. Proc. Soc. Exper. Biol. & Med. 92: 803–806, 1956.

(7) MCLIMANS, W. F., DAVIS, E. V., GLOVER, F., RAKE, A., and RAKE, G. W.: Submerged culture of mammalian cells—the spinner culture. (Abstract.) Federation Proc. 16: 425, 1957.

(8) MCQUILKIN, W. T., EVANS, V. J., and EARLE, W. R.: The adaptation of additional lines of NCTC clone 929 (strain L) cells to chemically defined protein-free medium NCTC 109. J. Nat. Cancer Inst. 19: 885–907, 1957.

(9) RINALDINI, L. M.: A quantitative method for growing animal cells *in vitro*. Nature, London, 173: 1134–1135, 1954.

(10) PUCK, T. T., MARCUS, P. I., and CIECIURA, S. J.: Clonal growth of mammalian cells in vitro; growth characteristics of colonies from single HeLa cells with and without a feeder layer. J. Exper. Med. 103: 273–284, 1956.

(11) YAMADA, M., and TAKANO, K.: Growth curve of HeLa strain cells in tissue culture. Jap. J. M. Sc. & Biol. 9: 27–39, 1956.

(12) SANFORD, K. K., EARLE, W. R., and LIKELY, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246, 1948.

(13) HOBBS, G. L., SANFORD, K. K., EVANS, V. J., and EARLE, W. R.: Establishment of a clone of mouse liver cells from a single isolated cell. J. Nat. Cancer Inst. 18: 701–707, 1957.

(14) GEY, G. O., BANG, F. B., and GEY, M. K.: Responses of a variety of normal and malignant cells to continuous cultivation, and some practical applications of these responses in the biology of diseases. Ann. New York Acad. Sc. 58: 976–999, 1954.

(15) GEY, G. O., COFFMAN, W. D., and KUBICEK, M. T.: Tissue culture studies of the proliferative capacity of cervical carinoma and normal epithelium. Cancer Res. 12: 264–265, 1952.

(16) PERRY, V. P., EVANS, V. J., EARLE, W. R., HYATT, G. W., and BEDELL, W. C.: Long-term tissue culture of human skin. Am. J. Hyg. 63: 52–58, 1956.

(17) EARLE, W. R., and HIGHHOUSE, F.: Culture flasks for use with plane surface tissue cultures. J. Nat. Cancer Inst. 14: 841–851, 1954.

(18) EVANS, V. J., BRYANT, J. C., MCQUILKIN, W. T., FIORAMONTI, M. C., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. III. Whole egg extract ultrafiltrate for long-term cultivation of strain L cells. Cancer Res. 17: 317–324, 1957.

(19) EVANS, V. J., BRYANT, J. C., FIORAMONTI, M. C., MCQUILKIN, W. T., SANFORD, K. K., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. I. A protein-free chemically defined medium for cultivation of strain L cells. Cancer Res. 16: 77–86, 1956.

(20) EVANS, V. J., BRYANT, J. C., MCQUILKIN, W. T., FIORAMONTI, M. C., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. II. An improved protein-free chemically defined medium for long-term cultivation of strain L 929 cells. Cancer Res. 16: 87–94, 1956.

(21) EARLE, W. R.: Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212, 1943.

(22) BRYANT, J. C., EARLE, W. R., and PEPPERS, E. V.: The effect of ultracentrifugation and hyaluronidase on the filtrability of chick-embryo extract for tissue culture. J. Nat. Cancer Inst. 14: 189–225, 1953.

(23) SANFORD, K. K., EARLE, W. R., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E., JR.: The measurement of proliferation of tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1951.

(*24*) SANFORD, K. K., WESTFALL, B. B., FIORAMONTI, M. C., MCQUILKIN, W. T., BRYANT, J. C., PEPPERS, E. V., EVANS, V. J., and EARLE, W. R.: The effect of serum fractions on the proliferation of strain L mouse cells *in vitro*. J. Nat Cancer Inst. 16: 789–802, 1955.

(*25*) GUNSALUS, I. C.: Growth of bacteria. *In* Bacterial Physiology (Werkman, C. H. and Wilson, P. W., eds.). New York, Academic Press, Inc., 1951, pp. 101–125.

(*26*) THIMANN, K. V.: Growth and protoplasm formation. *In* The Life of Bacteria. New York, The Macmillan Company, 1955, pp. 549–578.

(*27*) TOPLEY, W. W. C., and WILSON, G. S.: The growth and death of bacteria. *In* Principles of Bacteriology and Immunity, 3rd ed. Baltimore, The Williams & Wilkins Co., 1946, vol. 1, pp. 80–100.

(*28*) WESTFALL, B. B., PEPPERS, E. V., and EARLE, W. R.: The change in concentration of certain constituents of the medium during growth of the strain HeLa cells. Am. J. Hyg. 61: 326–333, 1955.

(*29*) WESTFALL, B. B., PEPPERS, E. V., BRYANT, J. C., SCHILLING, E. L., and EARLE, W. R.: Effect on the medium of growth of clone NCTC 929 cells as suspensions in agitated flasks with simple fluid addition. J. Nat. Cancer Inst. 21: 1958. In press.

(*30*) WESTFALL, B. B., EVANS, V. J., PEPPERS, E. V., HAWKINS, N. M., BRYANT, J. C., SCHILLING, E. L., and EARLE, W. R.: Observations of the metabolic behavior of a clone of mouse liver cells grown in agitated fluid suspensions. In preparation.

Massive Fluid-Suspension Cultures of Certain Mammalian Tissue Cells. II. Glucose Utilization and Cell Proliferation [1, 2, 3]

JAY C. BRYANT, EDWARD L. SCHILLING, *and* WILTON R. EARLE, *Tissue Culture Section, Laboratory of Biology, National Cancer Institute,*[4] *Bethesda, Maryland*

Summary

J. Nat. Cancer Inst. 21: 349–364, 1958

Utilization of glucose by several established strains of mammalian cells in large, agitated, fluid-suspension cultures under conditions of continuous aeration and agitation has been studied, particularly during the logarithmic phase of growth.

The rate of glucose utilization per million nuclei per day ranged from a maximum of 1.51 mg. to a minimum of 0.13 mg. Average rates of glucose utilization by cultures of established cell strains were: 0.86 mg. by human skin epithelial NCTC strain 1769, 0.56 mg. by mouse liver NCTC clone 1469, and 0.38 mg. by mouse fibroblast NCTC clone 929 (strain L). Comparable rates of use during slow growth were 0.71 mg. by 1 culture of high sarcoma-producing NCTC strain 1742, and 0.26 mg. by 1 culture of low sarcoma-producing NCTC strain 2049. The glucose utilization rate appeared to be a fairly constant characteristic of established cell strains when other conditions were uniform.

Glucose was generally used at a higher rate during the initial lag phase than during the logarithmic phase; the rate was higher during the early stages of the logarithmic phase than during the later stages of that phase. These trends and the frequently observed simultaneous trend toward lower pH suggest that considerable utilization of lactic or keto acids occurs in the later stages of the logarithmic phase.

In cultures of clone 929 cells receiving fresh fluid in thrice-weekly increments without removal of old fluid, a rapid decrease of glucose to exhaustion, in the medium, immediately preceded a sharp population decrease.

Glucose utilization studies with long-term tissue cultures of free-floating rapidly agitated cell suspensions under continuous aeration have not been reported previously in any detail. Short-term studies on the rela-

[1] Received for publication February 19, 1958.

[2] The authors wish to express their gratitude to Mrs. Evelyn Peake and Dr. W. C. Alford of the Microanalytical Laboratory, Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, who performed the chemical analyses.

[3] Much of these data were originally reported at the annual meeting of the Tissue Culture Association at Baltimore, Md., on April 16, 1957.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

tionship between glucose utilization and cell numbers, with tissue cultures in static flasks or in small roller tubes, have been made in this and other laboratories (*1–7*).

The present paper reports a study of glucose utilization by cells of several different established cell strains in agitated fluid-suspension cultures. Major emphasis has been placed on relationships between cell proliferation and glucose utilization, particularly duriug the logarithmic phase of growth. Certain factors related to manner and frequency of media renewal are also considered.

Materials and Methods

Cell strains.—This study was carried out on 7 established strains of cells. Five of these were also used in the previous study (*8*): NCTC clone 929 from mouse fibroblast strain L; NCTC clone 1469 from mouse epithelial cells of the parenchyma of the liver; NCTC strains 2071 and 2168, which had been adapted from NCTC clone 929 so that they could be maintained in reasonably rapid proliferation in protein-free, chemically defined medium NCTC 109, or in that medium plus 0.25 percent horse serum, respectively; and NCTC strain 1769 from human skin epithelium. The 2 additional cell strains used were high sarcoma-producing NCTC strain 1742 and low sarcoma-producing NCTC strain 2049, both of which had been developed from a single cell that had been cloned from normal, subcutaneous, adipose connective tissue of a strain C3H mouse (*9*).

Culture media.—The nutrient media used are indicated in table 1. Serum supplied large molecule components, and either whole egg extract ultrafiltrate or NCTC 109, with Earle's saline, supplied the small molecule components.

Glucose was furnished in the saline as dextrose.[5] Glucose concentration in fresh culture medium was at least 260 mg. percent. The egg ultrafiltrate or NCTC 109 components and the serum component had 100 mg. percent glucose, so that it was necessary for the remaining components of a given medium to carry a relatively high concentration of glucose. Accordingly, stock lots of 2 high glucose modifications of Earle's saline (*10*) were prepared periodically as sterile solutions containing glucose at either 500 or 2,000 mg. percent concentration. These salines were osmotically compensated on a theoretical basis by reducing the sodium chloride content. The high glucose-saline component always constituted 40 percent of the final medium by volume. The remaining 60 percent of the medium, *e.g.*, egg ultrafiltrate and serum, then diluted the 500 mg. percent glucose component to 260 mg. percent. When higher concentrations of glucose were used in the final medium, both the 2,000 mg. percent glucose saline and the 500 mg. percent glucose saline were combined in appropriate proportions in the 40 percent component.

Chemical analyses.—Reducing sugar was determined in 1 or 2 ml. of supernatant from a centrifuged 5 ml. sample of the cell suspension at each

[5] Dextrose, Anhydrous, Merck & Co., Inc., Blue Label Biological Grade is the same grade regularly used in all tissue-culture media in this laboratory.

fluid change, fluid addition, or subculture. The mild centrifugation at about 400 r.p.m. for 20 minutes did not injure the cells. The remainder of the 5 ml. sample, including all cells in it, was returned to the culture flask. The analytical method used was the Somogyi colorimetric copper-reduction method for blood sugar (*11*).

Reducing-sugar determinations were made regularly on all cultures at each fluid change or fluid addition from April, 1955, to date, *i.e.*, cultures in experiment 2122 and later experiments. In early experiments the aliquot sample was frozen immediately in dry ice and stored at −10° C. until the analysis could be performed, usually within 2 or 3 days. After September, 1955, that is, in all experiments after number 2244, the samples were usually analyzed within 1 hour after taking and centrifugation.

Basis for calculating glucose utilization.—The level of reducing sugar found in the old fluid of a given culture, when compared with the known concentration of glucose in the fresh medium at the preceding fluid change, gave a measure of the amount of glucose decrease per unit volume of fluid per unit time during the interval. Control flasks containing only fresh culture fluid were used in various experiments often enough to establish the fact that the glucose level remained relatively constant in a flask of agitated culture fluid without cells. It has been considered that reducing sugar is equivalent to glucose and that glucose decrease as defined represents actual glucose utilization by the cells. Glucose utilization is defined in these terms throughout this paper.

Composition of supernatant air in cultures.—The carbon dioxide content of the gas mixture used for continuous aeration of the agitated cultures was 5 percent. The oxygen content of the gas mixture was approximately 19 percent in all cultures with series numbers smaller than 2281. A gas mixture containing 40 percent oxygen was supplied to cultures 2281-1-1 and 2282-1-1 (described in table 5), 2303-1-1 (*see* footnote 7), and to cultures 2313-1-1, 2313-1-2, and 2320-1-2 (listed in table 1).

Estimation of p*H.*—The high bicarbonate component of Earle's saline (2.20 gm. $NaHCO_3$ per liter) renders any medium based on that saline, or on such other medium components as serum, dependent on variations in the carbon dioxide content of the overlying air. Phenol red was incorporated in the fresh medium at each fluid change as the sterile lyophilized form at 0.002 percent concentration. The *p*H of the old media in cell cultures was estimated at each fluid change by the following procedure.

About 15 minutes after the culture flask was disconnected from the continuous air-flow line in the shaker-incubator and taken into the sterile room, the fluid suspension was pipetted into 100 ml. or 200 ml. centrifuge bottles. Each bottle was then tightly stoppered and was centrifuged for 20 minutes at 400 to 900 r.p.m. in a #2 International centrifuge in order to effect separation of the cells. A 12 ml. portion of the centrifuged supernatant was pipetted directly from one of the centrifuge bottles into a 15 × 150 ml. test tube, which was tightly stoppered immediately. The color of the fluid in the tube was checked in a comparator block against the colors of 2 buffer solutions in tubes of the same size. The buffer solu-

tions consisted of appropriate proportions of M/15 Na_2HPO_4 and M/15 KH_2PO_4 to cover the *p*H range 6.6 to 8.0, mostly in 0.1 *p*H steps, and contained 0.002 percent phenol red. The comparator block also included 2 recently prepared, tightly stoppered correction tubes of serum, appropriately diluted with saline, to be lined up with each tube of buffer solution.

The chief limitation of this method was exposure of the high bicarbonate culture fluid to air and consequent tendency toward an alkaline shift before the *p*H was read. Such exposure for only a few minutes is ordinarily associated with an alkaline shift which can be perceived readily in small volumes of fluid containing .002 percent phenol red (2 to 3 ml.). In this study, larger volumes of fluid, during the time between removal of the flask from the air line and transfer from the centrifuge tube, did not show any perceptible color change. Much of this time the fluid was either in the culture flask still containing high CO_2 air or in tightly stoppered centrifuge flasks with a very small relative volume of air. The final transfer of the small volume of fluid to the comparator tube was so rapid and the volume of air in the tightly stoppered tube was so small that any perceptible color change did not occur. All fluid transfers in this sequence were accomplished by pipetting, which entailed a minimum of exposure to the air low in CO_2.

Other factors.—The equipment and methods for incubation and agitation, the methods of handling the cultures, and the short modified method for enumeration of nuclei in aliquot samples from the cell suspension have been described in earlier papers (*8*, *12*).

Experiments and Results

Glucose Utilization and Nuclei Population

Data on glucose utilization in relation to nuclei population in 9 representative cultures during individual fluid change intervals in the logarithmic phase of growth are presented in table 1. Quantitative and morphologic characteristics of the logarithmic phase of growth of these cultures have been outlined in the previous paper (*8*). The significant derived data are in the following 3 columns in table 1: B, mean numbers of nuclei, E, actual amount of glucose decrease in mg.,[6] and F, glucose decrease in mg. per million nuclei per day.[6] Column B gives the geo-

[6] The actual glucose decrease (col. E, table 1) in the entire fluid of the culture during each individual fluid change interval has been calculated by this empirical equation:

$$E = \frac{DC}{100}$$

Here E is actual glucose decrease (mg.), D is decrease in glucose concentration (mg. per 100 ml.), and C is fluid volume (ml.). The total glucose used by the culture during the logarithmic phase is the sum of the actual glucose decreases for all of the individual fluid change intervals.

Glucose decrease per million nuclei per day (col. F, table 1) expresses the relationship between glucose utilization and nuclei population. The difference in time duration between the 2-day and 3-day fluid change intervals is compensated by introducing the number of days in the denominator of the following empirical equation for glucose decrease per million nuclei per day:

$$F = \frac{E}{BA}$$

Here F is glucose decrease per million nuclei per day (mg.), B is mean number of nuclei, and A is number of days. The average glucose decrease per million nuclei per day during the logarithmic phase of a given culture is the average of the separate values for the individual fluid change periods.

metric mean of the numbers of nuclei at the start and at the end of a given period; the geometric mean is used as representing a closer approximation to the actual number of nuclei present during the entire period than the arithmetical average, particularly when the cells are proliferating rapidly.

One of the significant trends displayed in table 1 is that glucose utilization per million nuclei per day is usually highest at the beginning of the logarithmic phase, and then decreases regularly. This trend is clearly expressed, with the exceptions of the early part of the logarithmic phase of culture 2163-1-1 and the last periods of the logarithmic phase of cultures 2260-2-2 and 2260-2-4. Other experiments not listed in table 1 indicate that this trend is real. On the basis of this and other experimental data on hand, it is clear that both the consistent drop in glucose utilization rate (col. F) during the logarithmic phase and the termination of the logarithmic phase of growth are associated with factors other than exhaustion of glucose in the medium.

Table 2 summarizes group averages of glucose utilization rates for all cultures presented in table 1 of this paper,[7] and correlates them with corresponding group averages of proliferation of the same cultures that have been presented in table 1 of the previous paper. (*8*)[8] Table 2 shows that during the logarithmic phase, cultures of cells of human skin epithelium strain 1769 were characterized by comparatively high values for generation time,[9] total increase in numbers of nuclei, and total amounts of glucose used per culture; average rates of glucose utilization per million nuclei increase, and average rates of glucose utilization per million nuclei per day were also high. The cultures of human skin epithelial cells used more than twice as much glucose, per million nuclei per day, as did either line of clone 929 cells, and significantly more than did the liver clone 1469 cells. Average glucose utilization rate appears to be a specific characteristic of each established cell strain used in this study.

Addition of Fluid Increments

The complete fluid change usually carried out on the agitated fluid-suspension cultures involved considerable expenditure of time and labor. Centrifugation in particular required the sterile pipetting of comparatively large volumes of cell suspension and supernatant fluid. Much of this work could be avoided if increments of fresh fluid could be added periodically to the fluid suspension without first removing the old fluid. Experiment 2122 was started with cells of clone 929 to explore this possi-

[7] The 4 human skin cultures in table 2 include, in addition to the 2 cultures listed in table 1 of this paper, 2 other cultures, 2179-1-1 and 2303-1-1 listed in table 1 of the previous paper (*8*). The values for these 2 cultures are, respectively: total glucose decrease 5760 and 1154 mg., average glucose decrease per million nuclei increase 2.5 and 9.6 mg., and average glucose decrease per million nuclei per day 0.95 and 0.92 mg.

[8] The average values for the 4 human skin strain 1769 cultures in table 2 (*see* footnote 7) are derived from specific logarithmic phases of the following lengths as shown in table 1 of the previous paper (*8*): 28 days for culture 2179-1-1, 14 days for culture 2199-1-1, 16 days for culture 2303-1-1, and 7 days for culture 2320-1-2.

[9] "Generation time" here indicates average life span of an individual cell (*8*); this term must be clearly distinguished from the term "transfer generation" as used in this laboratory to indicate the period of maintenance of a culture as 1 entity in 1 culture flask or other container.

TABLE 1.—*Glucose utilization by representative suspension cultures during individual fluid change intervals in the logarithmic phase of growth*

Cell strain	Culture designation*	Components of culture medium†				Numbers of nuclei during logarithmic phase (*millions*)		Period between fluid changes					Glucose concentration in fresh fluid (*mg./100 ml.*)	Glucose decrease		
		Small molecule		Serum					A	B		C		D	E	F
		Per-cent	Kind‡	Per cent	Kind	At start n_1	Increase n_2-n_1	Se-quence§	Dura-tion (*days*)	Numbers of nuclei (*millions*)		Fluid vol-ume (*ml.*)		Concen-tration (*mg.-%*)	Amount	Per million nuclei per day (*mg.*)
										At end	Mean					
Human skin epithelium (NCTC strain 1769)	2199-1-1	20	WUF	40	Human	189.0	1782.6	10th	3	298.4	237.5	225	620	478	1076	1.51
								11th	2	425.3	356.2	275	380	203	558	0.78
								12th	2	462.3	443.4	335	380	188	630	0.71
								13th	3	676.0	559.0	440	380	233	932	0.56
								14th	2	831.6	749.7	425	380	205	871	0.58
								15th	2	1971.6	1280.5	400	380	230	920	0.36
									14						*4987*	*0.76*‖
	2320-1-2	20	WUF	40	Human	60.5	177.0	4th	2	99.7	77.7	100	260	191	191	1.23
								5th	3	162.5	127.3	125	380	270	338	0.89
								6th	2	237.5	196.5	175	260	76	133	0.34
									7						*663*	*0.82*‖
Mouse liver epithelium (NCTC clone 1469)	2163-1-1	20	WUF	40	Horse	10.3	1092.7	7th	2	ND	20.4	80	260	160	128	0.48
								8th	2	ND		100	260	54	54	
								9th	3	40.5		150	260	46	69	
								10th	2	78.7	56.5	150	260	54	81	0.72
								11th	2	127.8	100.3	175	260	50	88	0.44
								12th	2	283.3	190.3	200	260	88	176	0.46
								13th	2	501.1	377.3	225	260	152	342	0.45
								14th	2	1103.1	743.5	275	380	186	511	0.34
									17						*1449*	*0.48*‖
	2213-2-1	20	WUF	40	Horse	320.0	914.5	5th	2	492.0	396.8	450	380	174	783	0.99
								6th	2	593.3	540.3	400	260	154	616	0.57
								7th	3	1234.5	855.8	450	380	243	1094	0.43
									7						*2493*	*0.66*‖
	2213-3-1	20	WUF	40	Horse	534.0	424.5	3rd	3	627.3	578.8	450	380	220	990	0.57
								4th	2	958.5	775.4	450	260	183	824	0.53
									5						*1814*	*0.55*‖
Mouse fibroblast (NCTC clone 929)	2313-1-1	20	WUF	40	Horse	127.3	299.7	2nd	2	271.3	185.8	125	260	134	168	0.45
								3rd	3	427.0	340.4	300	320	94	282	0.28
									5						*460*	*0.37*‖
	2313-1-2	20	WUF	40	Horse	133.7	215.3	2nd	2	240.8	179.4	125	260	115	144	0.40
								3rd	3	349.0	290.0	300	320	109	327	0.38
									5						*471*	*0.39*‖

Mouse fibroblast (NCTC clone 929, strain (2168)	2260-2-2	40	NCTC 109	20	Horse	60.9	401.0	1st	3	146.2	95.0	100	260	88	88	0.31
								2nd	2	224.9	182.4	220	260	30	66	0.18
								3rd	2	461.9	322.3	338	260	60	203	0.31
									7						357	*0.27*‖
	2260-2-4	40	NCTC 109	20	Horse	96.2	400.0	1st	3	171.8	128.6	100	260	98	98	0.25
								2nd	2	243.4	204.5	175	260	50	88	0.22
								3rd	2	496.2	347.5	258	260	60	155	0.36
									7						*341*	*0.28*‖

*In this column the 1st number identifies the experiment (or series), the 2nd number, the transfer generation in that experiment, and the 3rd number, the specific culture in that generation.

†In addition to Earle's saline (*10*).

‡WUF = Whole egg ultrafiltrate, and NCTC 109 = protein-free chemically defined medium (*8*).

§Number of the specific fluid change from beginning of experiment.

‖Average of values for fluid change intervals.

TABLE 2.—*Proliferation and glucose utilization during the logarithmic phase of growth by representative suspension cultures of various cell strains*

Cell strain	Numbers of cultures	Average duration of logarithmic phase (*days*)	Nuclei generation time (*days*)	Average numbers of nuclei per culture (*millions*)		Average glucose decrease per culture*			
				At start logarithmic phase	Increase per culture	Total per culture (*mg.*)	Per million nuclei increase (*mg.*)	Per million nuclei per day (*mg.*)	Per 10^9† nuclei per hour (μM.)
Human skin epithelium NCTC 1769	4	16.3	4.60	74.0	1,097.6	3,141	4.65	0.86	199
Mouse liver epithelium NCTC clone 1469	3	14.5	4.02	288.1	810.6	1,919	2.78	0.56	130
Mouse fibroblast NCTC clone 929	2	5.0	3.25	130.5	257.5	461	1.85	0.38	88
Mouse fibroblast NCTC strains 2071 and 2168‡	2	7.0	2.68	78.6	400.5	349	0.87	0.275	64

*Glucose concentration in fresh fluid was usually 260 to 320 mg. percent.

†Leslie *et al.* (*7*) used this expression for glucose utilization rate.

‡Previously adapted to chemically defined medium NCTC 108 with 0.25 percent horse serum.

bility. Eight cultures were planted with about 70 million cells (range 53 to 83 million nuclei) in 100 ml. fluid in each flask. Fluid was added 3 times each week in uniform increments at 4 different levels: 25 ml., 35 ml., 50 ml., and 65 ml.; 2 cultures were carried on each fluid volume. Both the original inoculum of 100 ml. volume and the increments of fresh fluid contained 260 mg. percent glucose. The culture medium was 40 percent horse serum, 20 percent whole egg ultrafiltrate, and 40 percent high glucose saline.

Proliferation curves, fluid volumes, and glucose concentrations for the pair of cultures receiving 35 ml. increments of fresh fluid are shown in text-figure 1. The behavior of these cultures was essentially representative of the other pairs of cultures in the experiment. The rates of glucose utilization by each of the 8 cultures during each fluid addition interval are presented in table 3. Usually the logarithmic phase began after fluid addition on the 2nd day and was superseded by the stationary phase after fluid addition on the 11th day.

The major results of experiment 2122 may be stated as follows:

1) The lag phase was quite similar in duration and intensity at all levels of fluid addition within the range studied.

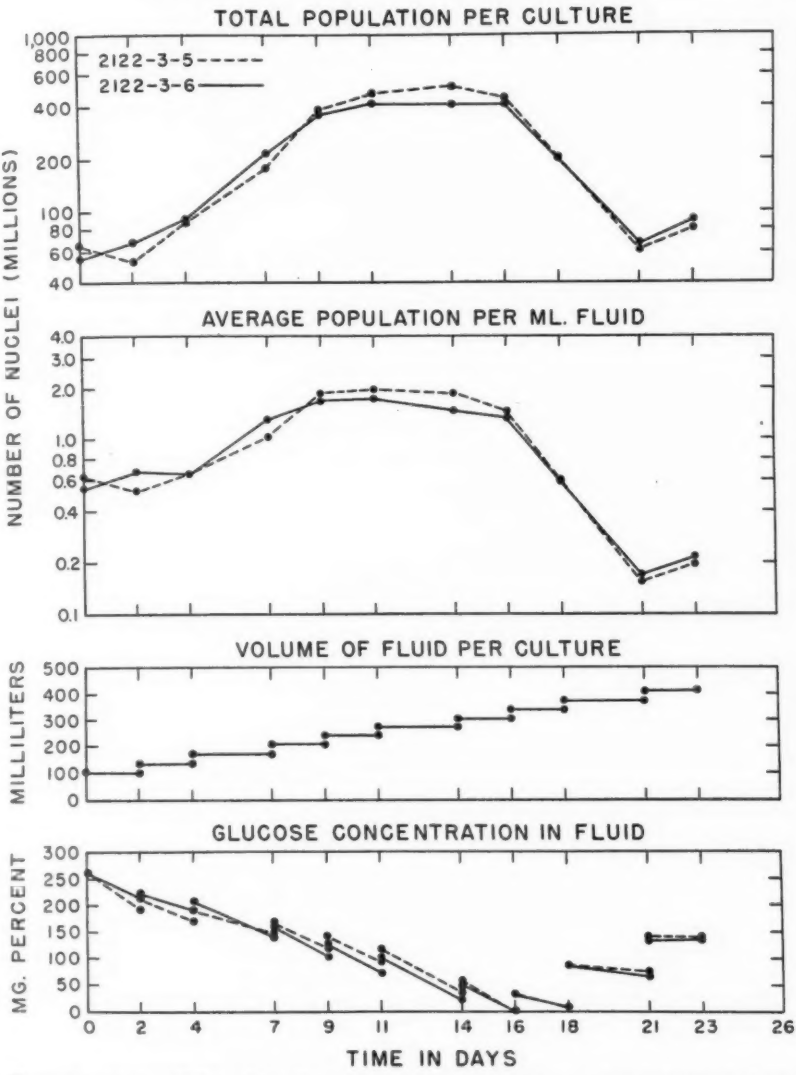
2) Within the logarithmic phase there was usually some reduction in generation time with larger fluid addition increments (table 4), *i.e.*, faster rate of increase. The logarithmic phase was approximately equal in length in all cultures except in those receiving the largest fluid addition increments (table 3).

3) The length of the stationary phase was quite variable between the cultures of different sets and tended to be longer in cultures that received smaller fluid addition increments.

4) The phase of decline in all cultures occurred when total volumes reached 345 to 425 ml. under the conditions of the experiment, and hence usually began later in the cultures receiving the smaller fluid increments.

5) The precipitous population decline followed closely the last stages of a long-continued decline in glucose concentration, in cultures receiving 35 ml. (text-fig. 1), 50 ml., and 65 ml. fluid increments. The fact that the cultures receiving 25 ml. increments did not show such a decline (table 3) was associated with a lower maximum cell population level during the stationary phase. This suggests that some unrecognized factor, possibly mechanical, was complicating results at this small increment volume level.

6) The rate of glucose utilization during each 2-day or 3-day fluid addition period (table 3) showed a consistent trend from relatively high values during the lag phase to lower values later. For example, cells in culture 5 used 0.58 mg. glucose per million nuclei per day during the initial 2-day lag period, and 0.40 mg. glucose per million nuclei per day during the next 2 days. During the remainder of the logarithmic phase, the stationary phase, and the phase of decline, glucose was used at rates of 0.20 mg. per million nuclei per day or less. All 8 cultures showed a similar trend.



TEXT-FIGURE 1.—Cell proliferation, fluid volumes, and glucose concentrations characterizing duplicate fluid-suspension cultures 2122-3-5 and 2122-3-6 of NCTC clone 929 cells. Each point on the curves represents a 35 ml. fluid addition. Cell proliferation values are actually derived from numbers of nuclei counted in aliquot samples.

Glucose Utilization Rates During Slow Growth of 2 Different Cell Lines Derived from the Same Single Cell

Sharply contrasting rates of glucose utilization were a major point of difference between a suspension culture of a high sarcoma-producing

TABLE 3.—*Rate of glucose utilization by individual suspension cultures of clone 929 cells in experiment 2122 during each fluid addition period (mg. glucose per million nuclei per day) at 4 levels of increment volume*

Growth phase	Fluid addition period		25 ml. increments		35 ml. increments		50 ml. increments		65 ml. increments		Average rate during one period
	Sequence	Duration (*days*)	Culture No. 3	Culture No. 4	Culture No. 5	Culture No. 6	Culture No. 7	Culture No. 8	Culture No. 9	Culture No. 10	
Lag	1st	2	0. 36	0. 31	0. 58	0. 40	0. 43	0. 57	0. 42	0. 46	0. 44
Logarithmic	2nd	2	0. 17	0. 46	0. 40	0. 29	0. 41	0. 45	0. 31	0. 39*	0. 36
"	3rd	3	0. 30	0. 30	0. 20	0. 26	0. 26	0. 04	0. 21	0. 25	0. 23
"	4th	2	0. 26	0. 21	0. 18	0. 21	0. 20	0. 30	0. 20	0. 14	0. 21
"	5th	2	0. 15	0. 16	0. 13	0. 17	0. 20	0. 24	0. 19	0. 19	0. 18
Stationary or decline	6th	3	0. 14	0. 14	0. 15	0. 17	0. 18	0. 19	0. 19†	0. 33‡	0. 19
"	7th	2	0. 15	0. 17	0. 18	0. 16	0. 23	0. 30‡	0. 19‡	0. 21‡	0. 20
"	8th	2	0. 07	0. 08	0. 13‡	0. 12‡	0. 02‡	0. 11‡	—	—	—
"	9th	3	0. 23	0. 22	0. 12‡	0. 22‡	—	—	—	—	—
"	10th	2	0. 24	0. 19	—	—	—	—	—	—	—
"	11th	2	0. 27	0. 23	—	—	—	—	—	—	—
"	12th	3	0. 24‡	0. 18	—	—	—	—	—	—	—
Average per culture			0. 22	0. 22	0. 21	0. 20	0. 24	0. 28	0. 25	0. 28	
Average per group			*0. 22*		*0. 205*		*0. 26*		*0. 265*		

*Lag phase in culture 10. †Logarithmic phase in culture 9. ‡Phase of decline.

TABLE 4.—*Proliferation and glucose utilization during the logarithmic phase of growth by 8 cultures of clone 929 cells receiving fluid additions (experiment 2122)*

Culture numbers	Volume of fluid increments (*ml.*)	Logarithmic phase in life of culture (*day*)			Nuclei numbers (*millions*)			Generation time (*days*)	Glucose decrease	
		t_1	t_2	(t_2-t_1)	n_1	n_2	n_2-n_1		Total amount (*mg.*)	Per million nuclei per day (*mg.*)
3	25	2	11	9	60.0	357.5	297.5	3.50	347	0.22
4	25	2	11	9	54.6	375.5	320.9	3.24	368	0.28
Total									*715*	
Average					*57.3*	*366.5*	*309.2*	*3.37*		*0.25*
5	35	2	11	9	52.0	466.9	414.9	2.84	327	0.23
6	35	2	11	9	66.2	414.3	348.1	3.40	400	0.23
Total									*727*	
Average					*59.1*	*440.6*	*381.5*	*3.12*		*0.23*
7	50	2	11	9	50.7	491.2	440.5	2.75	404	0.27
8	50	2	11	9	47.5	449.8	402.3	2.78	390	0.26
Total									*794*	
Average					*49.1*	*470.5*	*421.4*	*2.76*		*0.265*
9	65	2	14	12	66.5	820.5	754.0	3.31	751	0.22
10	65	4	11	7	57.5	430.0	372.5	2.41	308	0.19
Total									*1059*	
Average					*62.0*	*625.2*	*563.2*	*2.86*		*0.205*

cell strain NCTC 1742 and a culture of a low sarcoma-producing cell strain 2049, developed from the same single cell clone of mouse tissue (*9*). In this experiment the medium was 40 percent horse serum, 20 percent whole egg ultrafiltrate, and 40 percent high glucose saline, with glucose concentration at either 260 or 380 mg. percent. The 2 cultures increased slowly and rather irregularly in nuclei population. The concentration of glucose remaining in the old fluid at the fluid change, with one exception, was greater than 100 mg. percent, so that glucose should not have been a limiting factor at any time. Table 5 presents the trends of nuclei population and glucose utilization during the 26 days of the experiment. Although the nuclei population in the "low-line" culture was continuously greater than that in the "high-line" culture, total amount of glucose used by the former was appreciably less. Expressed in terms of average glucose decrease per million nuclei per day, the values are 0.26 and 0.71 mg., respectively. This consistent difference in glucose utilization between these 2 cell lines was also correlated with a consistent difference in *p*H of the culture fluid during the latter part of each fluid change interval: The high-line culture fluid generally gave a reading of 0.2 *p*H less than that of the low-line culture, *e.g.*, *p*H 6.9 and 7.1, respectively. It appeared that the relatively high metabolic activity of cells of the high sarcoma-producing line in fluid-suspension culture resulted in a relatively high degree of acid production.

Discussion

Two qualifications should be noted regarding the data for reducing sugar presented in this paper: 1) The assumption that the values for reducing sugar may be regarded as glucose neglects the background of reducing material which was not glucose and which probably represented 10 to 20 mg. percent in many samples. On this basis, a reducing-sugar analysis within this range could be interpreted to mean that no glucose remains. 2) It is assumed that the value for the amount of glucose in the fresh fluid, *e.g.*, 100 mg. percent or 260 mg. percent, represents the amount of glucose actually present in the culture at the beginning of the fluid change interval. This assumption is valid only for those instances when either an aliquot sample of the cell-free supernatant was taken without concomitant fluid change or when fresh fluid was added to the cell suspension and the glucose content of the resultant mixture could be calculated from a determination on an aliquot sample. In all other instances, *i.e.*, at fluid change or subculture, the soft pellet of cells and a few milliliters of old fluid remaining at the bottom of the centrifuge tube were returned, with an increment of the fresh fluid, to the culture flask. The cells and the old fluid thus diluted the fresh fluid slightly and lowered the concentration of glucose by a proportionate amount. Since the volume of cells and old fluid returned was usually only about 3 to 5 ml., or about 1 percent of a 400 ml. maximum volume, no correction has been made in the values of glucose in the fresh fluid. No reducing-sugar analyses were made on the fresh fluid either before or immediately after

TABLE 5.—*Glucose utilization and nuclei population in culture 2281-1-1 of a high sarcoma-producing cell strain and in culture 2282-1-1 of a low sarcoma-producing cell strain*

Fluid change period		Number of nuclei (*millions*)		Fluid volume (*ml.*)	Glucose in new fluid (*mg./100 ml.*)	Glucose decrease		
Number	Duration (*days*)	At start	Mean during period			Concentration (*mg./100 ml.*)	Total amount (*mg.*)	Per million nuclei per day (*mg.*)
High sarcoma-producing culture								
1	2	89. 0	52. 2	100	260	94	94	0. 90
2	3	30. 6	39. 8	50	260	146	73	0. 61
3	2	51. 7	47. 7	75	260	60	45	0. 47
4	2	44. 0	47. 0	75	260	120	90	0. 96
5	3	50. 3	55. 8	75	380	185	138. 8	0. 83
6	2	62. 0	57. 5	100	260	85	85	0. 74
7	2	53. 3	64. 8	75	260	122	91. 5	0. 71
8	3	78. 8	78. 2	100	380	162	162	0. 69
9	2	77. 7	85. 8	100	260	73	73	0. 43
10	2	94. 7	91. 2	125	260	118	157. 5	0. 86
11	3	87. 9	120. 8	100	260	230	230	0. 63
		166. 0						
Total	*26*						*1239. 8*	
Average		*73. 8*						*0. 71*
Low sarcoma-producing culture								
1	2	111. 3	107. 6	100	260	71	71	0. 33
2	3	104. 0	120. 1	100	260	56	56	0. 16
3	2	138. 7	133. 0	150	260	34	51	0. 19
4	2	127. 5	132. 2	150	260	63	94. 5	0. 36
5	3	137. 0	129. 8	150	380	117	175. 5	0. 45
6	2	123. 0	137. 5	175	260	40	70	0. 25
7	2	153. 6	197. 4	175	260	4	7	0. 02
8	3	253. 8	206. 0	225	380	92	207	0. 33
9	2	167. 2	200. 7	225	260	25	56. 3	0. 14
10	2	240. 8	142. 4	100	260	44	44	0. 15
11	3	84. 2	95. 5	100	260	139	139	0. 49
		108. 3						
Total	*26*						*971. 3*	
Average		*145. 8*						*0. 26*

the fluid change. Since the conditions of sampling and the method of analysis were uniform throughout this study, any error introduced by the 2 qualifications outlined may be considered to be minor and systematic.

Early in the present study it was found that glucose exhaustion, *i.e.*, very low levels of glucose in the medium for more than 1 or 2 days, appeared to have strongly inhibiting or destructive effects on cell proliferation in agitated fluid-suspension cultures, though the evidence was not conclusive. Consequently, the concentration in the media of most of the cultures in this study was increased to at least 2.6 times that generally used in tissue-culture media in this laboratory. Such concentrations were sufficient to prevent complete exhaustion of the glucose in the culture fluid during any fluid change interval.

Establishment of consistent differences in demand for, or utilization of, glucose by cells of different strains in large, agitated fluid-suspension culture constituted one of the major results of this study. Skin epithelial strain 1769 consistently utilized glucose at a high rate, liver epithelial clone 1469, at an intermediate rate, and clone 929, at a low rate. The difference between the glucose utilization rate of the high sarcoma-producing strain 1742 and that of the low sarcoma-producing strain 2049 was particularly striking, since the former was only slightly lower than that of the skin epithelium, while the latter was even lower than that of clone 929. Such consistent differences in rate of glucose utilization must of necessity indicate concomitant differences in metabolism.

Westfall, Evans, Shannon, and Earle (*1*) in this laboratory had found previously that mouse liver strain 721 cells (from a single cell of which clone 1469 (*13*) was originated) and mouse fibroblast clone 929 cells (originated from strain L) withdrew glucose from the medium in static replicate cultures at approximately the same rapid rate. The cells practically exhausted the 100 mg. percent glucose within 24 hours, so that during the remainder of the fluid change interval the source of energy for cell metabolism must have been something other than glucose. Since, however, the cultures of strain 721 in this study contained relatively fewer cells, there might actually have been a relatively high rate of glucose utilization by cells of that strain. In later experiments Westfall *et al.* (*2*) studied the effect of glutamine on glucose metabolism and cell growth in static cultures. Two closely related strains of mouse liver cells, clone 1469 and tumor strain 1795 (*14*) derived from clone 1469, were used. Under the conditions of the experiment both cell strains grew slowly in the presence of glutamine; in its absence both died. When the strains were compared for glucose utilization in the presence of glutamine, strain 1469 was using glucose at nearly twice the rate of strain 1795. Such differences in rate of glucose utilization between closely related cell strains are in complete accord with those found in the present study between unrelated cell strains.

Willmer (*4*) studied carbohydrate metabolism of chick-heart fibroblast and chick osteoblast cells in plasma and Tyrode's medium or plasma and embryo-extract medium in small flasks, static Carrel flasks, or slowly

rotating roller tubes, for 4 days or less. Initial concentration of glucose was either 100 or 200 mg. percent; fluid was usually changed at 48 hours. He found no absolute correlation between high proliferation rate and high glucose consumption. Westfall *et al.* (*1*, *2*) found no obvious connection between glucose utilization and cell proliferation, and concluded that sufficient information was not then available to establish firmly that differences in population density influenced the rate of glucose disappearance from the culture per unit number of cells per unit time. In the present study, however, most of the cultures reported in table 1 showed an inverse relationship between rate of glucose utilization and level of cell population or rate of proliferation during the logarithmic phase.

This inverse relationship agrees with the observation of Fulton, Sinclair, and Leslie (*6*) that total glucose utilization in slowly rotating roller-tube cultures of HeLa cells did not run parallel to cell population, increasing only about 10 percent for a fourfold increase in cell population. Expressed as amount of glucose utilized per unit number of cells per unit time, the rate further decreased with time as the culture grew. In their study, the cultures received daily changes of a medium consisting of 5 percent chick-embryo extract and 20 percent serum in modified Connaught medium 858 (*15*) with glucose at 100 mg. percent. Since culture fluid was changed every 24 hours, glucose concentration presumably did not fall to the exhaustion level, which characterized the cultures used by Westfall on which the fluid was changed only every 48 or 72 hours.

From these results of Fulton *et al.* (*6*) and the present study, it appears that when the complicating factor of severe depletion or complete exhaustion of glucose is eliminated, there is a well-defined inverse relationship between glucose utilization rate and level of cell proliferation during the lag and logarithmic phases of growth. Representative results from the present study outlined in table 3 show that in the stationary phase the glucose utilization rate remains substantially unchanged; in the phase of decline glucose utilization continues, but the relationship between glucose utilization and cell population level cannot be defined owing to inability to evaluate significantly the extent of cell deterioriation.

Fulton *et al.* (*6*) also determined the amounts of lactic acid and total keto acids produced in their cultures and found that the proportion of glucose appearing as these compounds diminished with the increase in cell population. They concluded that as glucose concentration in the medium lessened during a fluid change interval the energy requirements of the culture were met to a proportionate degree by utilization of lactic and keto acids. In a more recent paper (*7*) they reported that other cell strains, both human embryonic and malignant, showed a direct linear relationship between the cell number and the glucose utilized but not accounted for as lactic and keto acids. They suggested that the finding of previous investigators (*1*, *4*, *5*) in which there was no direct and consistent relationship between cell populations and glucose utilization might be explained by production of lactic and keto acids.

In the present study, while neither lactic nor keto acids were directly

determined, the lowest *p*H consistently characterized the fluid media in the most rapidly proliferating phase and probably indicated accumulation of lactic and keto acids even though glucose exhaustion was avoided. Oxygen, usually 19 percent and during one period 40 percent in the gas mixtures, supplied continuously to the cultures in this study was probably sufficient to allow reasonably efficient aerobic metabolism in any of the cultures reported; carbon dioxide (5%) in all such gas mixtures precluded any alkaline shift.

References

(*1*) WESTFALL, B. B., EVANS, V. J., SHANNON, J. E., JR., and EARLE, W. R.: The glycogen content of cell suspensions prepared from massive tissue culture: comparison of cells derived from mouse connective tissue and mouse liver. J. Nat. Cancer Inst. 14: 655–664, 1953.

(*2*) WESTFALL, B. B., EVANS, V. J., PEPPERS, E. V., HAWKINS, N. M., and EARLE, W. R.: Effect of glutamine on the growth and metabolism of liver cells *in vitro*. J. Nat. Cancer Inst. 17: 131–138, 1956.

(*3*) WESTFALL, B. B., PEPPERS, E. V., and EARLE, W. R.: The change in concentration of certain constituents in the medium during growth of the strain HeLa cells. Am. J. Hyg. 61: 326–333, 1955.

(*4*) WILLMER, E. N.: Carbohydrate metabolism of chick fibroblasts *in vitro*. J. Exper. Biol. 19: 237–256, 1942.

(*5*) WILSON, H., JACKSON, E. B., and BRUES, A. N.: Metabolism of tissue cultures. I. Preliminary studies on chick embryo. J. Gen. Physiol. 25: 689–703, 1942.

(*6*) FULTON, W. C., SINCLAIR, R., and LESLIE, I.: Changes in glucose utilization with the growth of tissue cultures of HeLa cells. (Abstract.) Biochem. J. 63 (part 2): 18P, 1956.

(*7*) LESLIE, I., FULTON, W. C., and SINCLAIR, R.: The metabolism of human embryonic and malignant cells and their response to insulin. Biochim. et biophys. acta 24: 365–380, 1957.

(*8*) BRYANT, J. C., SCHILLING, E. L., and EARLE, W. R.: Massive fluid-suspension-cultures of certain mammalian tissue cells. I. General characteristics of growth and trends of population. J. Nat. Cancer Inst. 21: 331–364, 1958.

(*9*) SANFORD, K. K., LIKELY, G. D., and EARLE, W. R.: The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells *in vitro*. J. Nat. Cancer Inst. 15: 215–237, 1954.

(*10*) EARLE, W. R.: Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212, 1943.

(*11*) SOMOGYI, M.: A new reagent for the determination of sugars. J. Biol. Chem. 160: 61–73, 1945.

(*12*) EARLE, W. R., BRYANT, J. C., SCHILLING, E. L., and EVANS, V. J.: Growth of cell suspensions in tissue culture. Ann. New York Acad. Sc. 63: 666–680, 1956.

(*13*) HOBBS, G. L., SANFORD, K. K., EVANS, V. J., and EARLE, W. R.: Establishment of a clone of mouse liver cells from a single isolated cell. J. Nat. Cancer Inst. 18: 701–707, 1957.

(*14*) EVANS, V. J., HAWKINS, N. M., WESTFALL, B. B., and EARLE, W. R.: Studies on culture lines derived from mouse liver parenchymatous cells grown in long-term tissue culture. Cancer Res. 18: 261–266, 1958.

(*15*) HEALY, G. M., FISHER, D. C., and PARKER, R. C.: Nutrition of animal cells in tissue culture. X. Synthetic medium no. 858. Proc. Soc. Exper. Biol. & Med. 89: 71–77, 1955.

Analysis of *W*-Series Pleiotropism in the Mouse: Effect of W^vW^v Substitution on Definitive Germ Cells and on Ovarian Tumorigenesis [1, 2]

ELIZABETH S. RUSSELL *and* ELIZABETH FEKETE,
Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

Summary

J. Nat. Cancer Inst. 21: 365–381, 1958

In the ovaries of C57BL-W^vW^v females, very few ovarian follicles are present at any time and these cease to grow and develop after the age of 2 months. Young and atretic follicles are found in diminishing numbers until 5 months.

The disappearance of follicles is accompanied by invagination of the germinal epithelium, culminating by the 7th month in the formation of tubular adenomas in all ovaries. These tumors later invade surrounding adipose tissues.

The small numbers of germ cells in these adult W^vW^v females, known to have developed from embryos with very few primordial germ cells, support the hypothesis that the definitive generation of germ cells is derived directly from the primordial generation.

At all ages, C57BL-W^vW^v females appear to have very little circulating estrogen, evidenced by small immature uteri and by absence of mating behavior.

Tumorigenesis in the sterile ovaries of C57BL-W^vW^v females is compared with that following X irradiation or splenic implantation of normal ovaries. Downgrowth of germinal epithelium precedes tumor formation in all three situations. Absence or a very low level of circulating estrogen, characteristic of all three situations, seems to be essential for production of ovarian tumors. Lack of developing ovarian follicles appears to cause the estrogen deprivation of W^vW^v females and probably also of females with X-irradiated ovaries. Destruction of estrogen in the liver is responsible for the low level of circulating estrogen in castrate females bearing splenic ovarian implants in areas drained by the hepatic portal system. Possible implications of differences in order of events leading toward tumor formation in each situation are discussed.

Genes with clear-cut effects that can be traced back into early developmental stages and forward to the end of an animal's life may provide a valuable mechanism for tracing the origin, course, and ultimate results of processes affected by their presence. This has proved true for genes

[1] Received for publication March 10, 1958.

[2] This investigation was aided by grants E-12 and E-76 to the Jackson Memorial Laboratory from the American Cancer Society, Inc., and by grant C-1073 from the National Institutes of Health, Public Health Service.

of the *W*-series in the house mouse, with well-defined effects upon viability, erythrocyte size and number, coat pigmentation, and fertility (*1*). Analyses of these pleiotropic effects have yielded fundamental information on hematopoiesis (*1, 2*), on melanoblast distribution (*3*), and on the origin of the primordial germ cell (*4*). The last-named study demonstrated a deficiency in germ-cell number in mice of three potentially sterile genotypes (*WW*, *WW*^{v}, and *W*^{v}*W*^{v}) as early as the 10th day of embryonic life, before the primordial germ cells reached the gonadal ridge. Between the 9th and 12th days of embryonic life the germ-cell numbers in normal embryos increase 10 to 30 times, while those in the potentially sterile embryos remain approximately constant. Thus the gonads of these defective types develop without the incorporation of appreciable numbers of primordial germ cells. Histological study of the ovaries and testes of normal and genetically sterile animals collected 0 to 28 days postnatal has demonstrated that the few ova and spermatogonia found in gonads of *WW*^{v} and *W*^{v}*W*^{v} animals develop more slowly than do the much larger numbers in the gonads of normal litter mates (*5*). At these immediate postnatal stages the germinal epithelium of the defective ovaries is also somewhat different from that of normal ovaries (*5*).

The present study, recording the later history of ovaries from mice of the most viable of the sterile genotypes, *W*^{v}*W*^{v}, is intended as a contribution to the determination of long-term effects of *W*-gene substitution, mediated through effects upon the primordial germ cells and possibly also through later action of these genes in or on ovarian tissues. Since all the older *W*^{v}*W*^{v} females have ovarian tumors, classified as tubular adenomas, this investigation may also be considered as a contribution to knowledge of the mechanism of tumorigenesis in mouse ovaries.

Material and Method

The mice used in this study were essentially isogenic, except for genes at the *W*-locus, with mice of strain C57BL/6. They were offspring of matings between *W*^{v}*w* parents from the 8th and later generations of backcrossing of *W*^{v} onto the C57BL/6 background. Ovaries from a total of 32 *W*^{v}*W*^{v} females were studied; 2 mice were sacrificed each month from 2 to 14 months of age, plus 6 sacrificed at ages between 15 and 25 months. Twenty-two of these mice were virgins, and 10 were kept with males for periods of 5 to 9 months.

The ovaries were sectioned serially and stained with hematoxylin and eosin. The volume of each ovary in mm.^{3} was estimated from measurement of sections. The presence and approximate number of primary, large, and atretic follicles were recorded for each ovary, as well as the condition of the germinal epithelium and ovarian stroma. The uteri of all animals were examined grossly and many of them were cross-sectioned and studied microscopically.

Results

Histology

Observations of the ovaries are given in concise form in table 1, and in more detail below.

Two months.—The ovaries of the 2-month-old animals were very small. The germinal epithelium in some regions showed slight invaginations and in others the cells were densely crowded together. There were several primary as well as atretic follicles, and the follicular cells of some of the atretic follicles were hypertrophied. A total of 3 large follicles with antra was found in the ovaries of both females, but the ova in each showed signs of degeneration (fig. 1).

Three months.—The ovaries of these animals were also small. The germinal epithelium showed considerable invagination and nowhere appeared crowded. There were primary and atretic follicles near the surface, and the atretic follicles showed varying degrees of degeneration, some containing only a hyalin clump as residue of the degenerated ovum.

Four months.—The ovaries at this age were slightly larger. The germinal epithelium formed deeper tubular invaginations, which branched in their terminal portions. Only a few very small follicles and some hyalin clumps (remains of ova from atretic follicles) were observed. The medulla of one ovary contained many hypertrophied cells, and small groups of such cells were also seen in the cortical zones (fig. 2). These cells resembled luteal cells and were considered to be hypertrophied stromal cells.

Five and 6 months.—These ovaries were considerably larger than those found in younger animals. The size increase was due to proliferation of the germinal epithelium and to increase in hypertrophied cells. The tubular invaginations of the germinal epithelium penetrated more deeply and branched more extensively. There were no normal follicles, though the ovaries of the 5-month-old animals contained a few hyalin clumps. Groups of hypertrophied cells were separated by the cuboidal epithelium of the branching tubules.

Seven and 8 months.—These ovaries were markedly larger than any of the earlier ones. No follicles or hyalin clumps were seen. The glands consisted entirely of branching tubules that divided them into intricate patterns, outlined by cuboidal epithelium and filled by hypertrophied luteal-like cells. A delicate stroma, rich in capillary network, partitioned the hypertrophied cells into small groups (fig. 3). It was considered that the progressive changes observed in these ovaries justified their classification as tubular adenomas. Invasion of the adjacent adipose tissue occurred at the hilus in all the ovaries.

Nine, 10, and 11 months.—The ovarian tumors of these animals were similar in size and structure to those found at 7 and 8 months. The invasion of the surrounding adipose tissue was more prominent. Both epithelial tubules and hypertrophied luteal-like cells had contributed to this invasion (fig. 4).

TABLE 1.—*Summary of histological findings in ovaries of C57BL-W•W• females—2 females sacrificed per month from 2 to 14 months, plus 6 females between 15 and 25 months, as indicated*

Age at sacrifice (*months*)	Time with male (plugs) (*months*)	Approximate volume ovary ($mm.^3$)	Follicles			Ovarian tumor
			Large	Primary	Atretic	
2	0	0. 15	2	Several	Several	No
	0	0. 13	1	Several	Several	No
3	0	0. 14	0	Several	Several	No
	0	0. 23	0	Few	Few	No
4	0	0. 25	0	Few	Few	No
	0	0. 40	0	0	0	No
5	0	0. 23	0	Few	Few	No
	0	0. 60	0	0	Few	No
6	0	0. 50	0	0	1	No
	0	0. 50	0	0	0	No
7	0	1. 10	0	0	0	Tubular adenoma
	0	1. 00	0	0	0	Tubular adenoma
8	5 (0)	1. 90	0	0	0	Tubular adenoma
	0	1. 80	0	0	0	Tubular adenoma
9	6 (0)	1. 70	0	0	0	Tubular adenoma
	6 (0)	1. 70	0	0	0	Tubular adenoma
10	6 (0)	1. 00	0	0	0	Tubular adenoma
	0	1. 50	0	0	0	Tubular adenoma
11	0	3. 60	0	0	0	Tubular adenoma
	9 (1)*	3. 50	0	0	0	Tubular adenoma
12	0	0. 70	0	0	0	Tubular adenoma
	6 (0)	1. 60	0	0	0	Tubular adenoma
13	6 (0)	1. 00	0	0	0	Tubular adenoma
	7 (0)	0. 80	0	0	0	Tubular adenoma
14	7 (0)	1. 40	0	0	0	Tubular adenoma
	6 (0)	2. 10	0	0	0	Tubular adenoma
15	0	2. 90	0	0	0	Tubular adenoma
16	0	2. 00	0	0	0	Tubular adenoma
17	0	2. 30	0	0	0	Cysts, tubular adenoma
18	0	3. 00	0	0	0	Cysts, tubular adenoma
21	0	2. 70	0	0	0	Cysts, tubular adenoma
25	0	4. 00	0	0	0	Cystic adenoma

*Vaginal plug at 4 months, no offspring produced.

Twelve, 13, and 14 months.—The ovarian tumors of these animals contained few hypertrophied cells and were more dense and cellular. The tubular adenomatous structure was evident (fig. 6). Invasion of the surrounding tissue occurred at both poles of the ovaries (fig. 5).

Older animals.—The ovaries of only 1 female were examined at each later stage. Tubular adenomas with dense stroma were seen in the ovaries of the 15- and 16-month-old females. The tubular adenomas of the ovaries of 17-, 18-, and 21-month-old females contained small multiple cysts. The 25-month-old female had a large cystic adenoma of the ovary (fig. 7).

To summarize briefly, the following changes take place in the ovaries of C57BL-W^vW^v females: The ovaries of 2- to 4-month-old W^vW^v animals are extremely small, but the over-all size increases progressively with advancing age. Ovarian follicles cease to grow after the mice reach the age of 2 months. Young and atretic follicles are present in diminishing numbers until the animals are 5 months old. The disappearance of the follicles is followed by deeper invaginations of the germinal epithelium. By the 7th month branching tubules formed by the ingrowing epithelium penetrate the ovaries completely and form tubular adenomas. The surrounding adipose tissue is invaded. At old age, cystic enlargement of the adenomatous structure may take place.

Test for fertility.—Ten of the females included in this study were tested for fertility (table 1). At weaning age these were placed with known-fertile males, and were kept with them for periods of 5 to 9 months. They were examined daily for vaginal plugs. Only 1 plug was observed in a female aged 4 months, who did not produce offspring. The estrogen level in most of these females evidently was not sufficient to produce estrus.

Condition of uteri.—Gross examination of the uteri of all animals showed them to be small and anemic. In cross sections the lumina were slit-shaped and the uterine glands were small, comparable to those of immature mice.

Discussion

Study of the ovaries of adult W^vW^v females may help to answer two types of theoretical questions: on the origin of the definitive generation of germ cells in the ovary, and on the causes of ovarian tumorigenesis.

The question whether both ova and follicular cells are formed throughout reproductive life by the germinal epithelium is still contested. Allen (*6*), as a result of extensive study of ovaries of mice, came to the conclusion that the germinal epithelium gives rise to both types of cells by processes of proliferation and differentiation, and that these processes continue during sexual maturity. Everett (*7*) suggested that the origin of the primordial germ cells of the mouse is extragonadal, and that after migrating to the genital ridge they become located among the somatic cells of the epithelium. He believed further that the ova which form in post-embryonic development are derived from these incorporated primordial germ cells. These germ cells are in such close relation to the somatic epithelial cells that it is difficult to differentiate them morphologically. He felt that the follicular cells arise from the somatic epithelial cells, and are carried into the cortex with the enlarged oocytes. As indicated earlier, the evidence from studies of *WW*, WW^v, and W^vW^v embryos (*4*)

supports completely Everett's hypothesis of the origin of primordial germ cells. The present observations also fit nicely with Everett's hypothesis of derivation of definitive germ cells from incorporation of primordial germ cells among the somatic cells of the germinal epithelium. Females of a genotype (W^vW^v) deficient in primordial germ cells as embryos also contain very few developing follicles with ova as adults. They show progressive reduction in this number with advancing age. The observations can be made compatible with Allen's hypothesis that the germinal epithelium of the adult gives rise to ova by assuming that *W*-gene action, which inhibits the multiplication of primordial germ cells in 9- to 12- day embryos, also affects the germinal epithelium independently, inhibiting its cells from differentiating into ova. Such dual action is improbable but not impossible, since independent action of *W*-genes in different tissues apparently occurs in connection with some of the various pleiotropic effects (*1*). Although evidence from the histology of adult W^vW^v ovaries can thus be made to fit with either of two opposing theories of the origin of the definitive generation of germ cells, it appears by the principle of Occam's razor more probable that Everett is correct in stating that ova in postpartum life are derived from distinct germ cells and not from somatic cells of the germinal epithelium. The gradual disappearance of developing follicles with advancing age suggests that a considerable population of germ cells may be necessary to maintain prolonged oogenesis.

Whatever may be the cause inhibiting the development of ovarian follicles in adult W^vW^v females, it does not decrease the proliferative potentiality of the germinal epithelium. Indeed, proliferation in adult life seems excessive, although this appearance may result in part from the accumulation of cells that remain as germinal epithelium rather than differentiating into follicular cells. The absence of germ cells in W^vW^v ovaries may contribute in either or both of two ways to the observed overgrowth and lack of differentiation of germinal epithelium. The presence and downward migration of enlarging oocytes may be essential for the differentiation of germinal epithelium and for organization of follicles. Secondly, without follicular organization estrogen may not be secreted, and this hormone may control proliferation of the germinal epithelium, either directly or through reciprocal relation with the pituitary. All the 22 ovaries recovered from C57BL-W^vW^v females 7 or more months old were classified as tubular adenomas. The tumors were hormonally inactive, but showed progressive growth and invasion of adjacent tissue.

Incidence of spontaneous ovarian tumors is low in all inbred strains of mice but higher in some than in others (*8*). Two different experimental procedures greatly increase incidence of ovarian tumors: X irradiation and transplantation of ovaries into the hepatic portal circulation of castrate hosts [*review* (*9*)].

It has long been known that tumors develop in the ovaries of mice exposed to X radiation. Brambell, Parkes, and Fielding (*10, 11*) described histological changes in ovaries of mice exposed to a sterilizing dose of X ray at 3 weeks postnatal or before birth. They reported com-

plete degeneration of the oocytes, followed by proliferation of cords of germinal epithelium penetrating into the medullary region. Furth and Butterworth (*12*) subjected young adult mice to sterilizing doses of X ray and several months later found numerous cases of tubular adenomas, apparently developed by invagination of the germinal epithelium, as well as other types of ovarian tumors. In general, although some ovarian tumors were hormone-secreting, the tubular adenomas were not. Ovarian tumors following X irradiation have since been reported in numerous inbred strains and F_1 hybrids (*13–15*) including C57BL (*16*). The minimum effective dose reported is very low, 50 r (*17*) or 87 r (*14*), and the total accumulated dose rather than duration of treatment is the deciding factor in tumorigenesis following irradiation (*17*). Mühlbock (*16*) reported a predominance of tubular adenomas and luteomas among tumors induced by radiation of C57BL ovaries, in contrast to granulosa-cell tumors found frequently in other strains. Eschenbrenner and Miller (*18*) summarized the histological changes leading to tumor formation in ovaries of LAF_1 females exposed to radiation. They found disappearance of follicles, downgrowth of germinal epithelium, and formation of ovarian tumors, some of which were tubular adenomas.

Induction of ovarian tumors in the mouse by transplantation of the ovary into the spleen of castrate hosts was first reported by Li and Gardner (*19*). Tumors were restricted to cases of clean operations without adhesions, which would allow escape of estrogens into the general body circulation. If there were adhesions in host females, the constitution of ovarian tissues remained relatively normal, with developing follicles and corpora lutea. In those without adhesions, the types of tumors observed (granulosa-cell tumors and luteomas) seemed dependent upon the sex of the castrate hosts, and most tumors appeared more than 200 days after splenic implantation, later in female than in male hosts. Intrasplenic ovaries recovered from animals sacrificed before this time almost all contained small numbers of primary or secondary ovarian follicles, some contained large follicles, and those recovered near the time of tumorigenesis also showed downgrowth of germinal epithelium. The authors suggest that the tumors, dependent upon the hormone imbalance, arise from these epithelial downgrowths. They also attribute to the same source the ova as well as the follicle cells seen in splenic transplants. Developing follicles were not found in intrasplenic transplants of irradiated ovaries, but this difference did not alter the incidence or time of appearance of ovarian tumors (*20*). Additional studies by Li and Gardner (*21*) added C57BL to the list of strains in which intrasplenic transplant of ovaries leads to formation of ovarian tumors, classified as granulosa-cell tumors and luteomas. Hummel (*22*) reported that ovarian tumors occurred in ovaries of C57BL females 5 months after splenic implantation. Several investigators have added critical evidence of the importance of reduction in circulating estrogen or increase in pituitary gonadotrophin for the induction of ovarian tumors (*9*). Tumorigenesis after intrasplenic transplant is prevented if an intact ovary remains *in situ*, or

if estrogens or androgens are added (*21*). There is direct evidence from parabiosis experiments that pituitary gonadotrophins are actually increased in castrate mice bearing intrasplenic ovarian transplants (*23*). The importance of increased pituitary gonadotrophins for production of ovarian tumors in intrasplenic transplants is indicated by Mühlbock's finding (*24*) of especially early tumor formation in ovaries implanted into the spleen of one of the members of a castrate parabiotic trio.

Reduction of circulating estrogen is also important in tumorigenesis following X irradiation. This has been demonstrated by the results obtained when only 1 of the 2 ovaries of BALB/c females was irradiated (*15*). Under this condition the irradiated ovary became small and anovular, but did not develop tubular downgrowths nor become tumorous. Intramuscular implants of irradiated ovaries into ovariectomized female hosts showed a high percentage of tumors, while implants of irradiated ovaries into intact female hosts showed none (*25*). Parabiosis experiments gave direct evidence that pituitary gonadotrophins were actually increased in mice with X-irradiated ovaries (*24, 26*). Kirschbaum, Liebelt, and Fletcher (*27*), however, obtained ovarian tumors at approximately the same rate with and without elevation of pituitary gonadotrophin when irradiated ovaries were implanted subcutaneously into intact (normal gonadotrophin) and castrate (elevated gonadotrophin) BALB/c males, suggesting ". . . it is possible that estrogenic hormone may act directly on the ovary to suppress tumor development."

There are many similarities, including dependence upon some type of hormone imbalance, between the causes of ovarian tumorigenesis following irradiation and following intrasplenic transplantation, but it has been suggested (*17, 25, 28, 29*) that radiation may have a direct tumorigenic effect upon the ovary in addition to the indirect effect through elevation of pituitary gonadotrophin. Some aspects of the role of diminution in numbers of ova may not have been sufficiently investigated. It is hoped that studies of ovarian tumorigenesis in a situation where lack of germ cells is the first observable deviation from normal may contribute to understanding of each of the ovarian tumor-promoting situations.

A comparison of ovarian tumorigenesis in C57BL-W^vW^v females with the processes in X-irradiated ovaries and in ovaries transplanted into the spleen may be helpful to summarize the similarities and differences between this situation and those previously reported:

1) The level of circulating estrogen is very low in C57BL-W^vW^v, as in all mice in which ovarian tumors develop. W^vW^v females differ, however, from the previously reported types in that there is no pretreatment stage of their life history in which they have had a normal estrogen level.

2) In all three situations, the pituitaries of animals developing ovarian tumors have normal potentialities, but are reacting to abnormal conditions. Although the deficiency of estrogen in C57BL-W^vW^v females may lead eventually to an increase in pituitary gonadotrophin, the pituitaries of young W^vW^v females are capable of responding normally to estrogenic stimulus. When ovaries from normal C57BL-*ww* females were implanted

into the ovarian capsules of weaning-age W^vW^v females, the recipients mated and produced and reared healthy normal litters, indicating normal reaction of the W^vW^v pituitary to estrogens from the implanted ovary, and normal response of uterus and mammary gland to pituitary stimulation (*30*, *31*).

3) W^vW^v ovaries have no stage at which there is a normal number of ova; they are deficient in number of germ cells from the time of their first organization in the embryo. Ovaries subjected to X irradiation or splenic implantation had large numbers of ova prior to treatment.

4) The few follicles found in young W^vW^v ovaries were frequently atretic and rarely developed beyond the primary or secondary follicle stage. Before treatment, X-irradiated or splenic-implanted ovaries contained many follicles in all stages of maturation. Cessation of follicular development appears to be an immediate result of heavy doses of X radiation, a greatly delayed result of doses as small as 50 r (*17*). After splenic implantation, follicular development continues for some time. In transplants without circulating estrogen, a few large as well as small follicles have been observed up to 250 days after operation (*21*), though their number and degree of development decreased with time.

5) The germinal epithelium of C57BL-W^vW^v ovaries showed extensive proliferation, with invaginations appearing as early as 3 months. Later deep branching tubules developed. The tubular invaginations increased as the ovarian follicles disappeared. Downgrowth of germinal epithelium is also associated with disappearance of follicles following irradiation (*18*) and splenic implantation (*19*). It does not occur if the estrogen level is high (*15*, *21*, *25*). In each situation, downgrowth of germinal epithelium is followed shortly by the appearance of ovarian tumors.

6) All the tumors found in C57BL–W^vW^v females from 7 to 21 months of age were classified as tubular adenomas; that in a 25-month-old female, as a cystic adenoma. Ovarian tumors found in other situations may be of several types, and mixed tumors are common. Those found following X irradiation include granulosa-cell tumors, luteomas, and tubular adenomas. C57BL may be susceptible to different types of tumors than other inbred strains, since those induced in this strain by X irradiation (*16*) were predominantly tubular adenomas and luteomas. However, the ovaries of C57BL females, as well as those from other inbred strains, produced both granulosa-cell tumors and luteomas when transplanted into the spleens of castrate hosts (*21*).

7) The W^vW^v genic substitution that is in all body cells may affect tissues other than ova in ways which alter tumorigenesis. A difference in other tissues that should be mentioned, although there is no evidence of pertinence in this context, is the severe macrocytic anemia characterizing all W^vW^v animals.

The finding of 100 percent ovarian tumors in C57BL–W^vW^v females 7 or more months old confirms the previous finding that absence or very low level of circulating estrogen promotes tumor formation. In W^vW^v and X-irradiated ovaries, the apparent reason for lack of estrogen is that

there are few or no developing ovarian follicles, and little or no estrogen is secreted. In the case of ovarian transplantation to the spleen, follicles in the implanted ovary continue for some time to enlarge, and estrogen is probably secreted but is destroyed in the hepatic portal system and does not reach the general circulation. In all three tumorigenic situations, typical histological findings in ovaries recovered shortly before the time of first appearance of ovarian tumors include absence of ovarian follicles and presence of tubular invaginations of the germinal epithelium. The order of events leading toward ovarian-tumor formation seems the same in W^vW^v ovaries and in normal ovaries subjected to X irradiation: small number of developing follicles, leading to low level of circulating estrogen; invagination of germinal epithelium; and, finally, tumor formation. The order is different in normal ovaries transplanted to the spleen of castrates: low level of circulating estrogen, followed by gradual diminution in the number of developing follicles; invagination of germinal epithelium; tumor formation.

It is possible that the suspected direct tumorigenic effect of radiation on the ovary is the elimination of some or all of an irreplaceable supply of definitive germ cells. Mouse oocytes degenerate very rapidly after heavy X irradiation (*32*). It may be that small numbers of ova are killed by direct hits with light doses of X radiation. The finding that total dose of radiation, however given, is the deciding factor in radiation tumorigenesis fits well with this theory. It is interesting to note that LAF_1 females given 50 r of X radiation produced normal numbers of normally sized litters throughout their breeding period, but later became anovular at a stage when untreated females still had many developing follicles (*17*). It is probable that reduction in number of developing follicles and decrease in circulating estrogen came very late in life because it was only then that the number of germ cells reached a critically low level. The appearance of tumors followed shortly after the anovular stage.

Loss of definitive germ cells through operation trauma may also contribute in some degree to tumorigenesis following splenic transplantation. Hummel (*22*) suggests reduction in amount of functional ovarian tissue as contributing to the ovarian tumorigenesis observed with some frequency following the double operation of ovarian transplantation into the spleen, followed after a short time by retransplantation to the ovarian capsule. The loss of ovarian tissue through a single transplant operation of ovary into spleen is not, however, sufficient to promote ovarian-tumor formation if estrogen escapes into the general body circulation.

Increased pituitary gonadotrophin resulting from lack of circulating estrogen is presented to a different array of ovarian cells in the 3 tumor-promoting conditions discussed here. In addition to possible differences in interstitial cells, there certainly are differences in the follicular cells. In these C57BL-W^vW^v females only 1- or 2-layered follicles could be influenced by elevated pituitary gonadotrophin. In X-irradiated ovaries, these would be present, plus, possibly, remains of degenerating many-

layered follicles or corpora lutea present before X irradiation. In intrasplenic ovaries, cells from many-layered follicles would be subjected to elevated pituitary gonadotrophic stimulation for long periods after transplantation. These differences in follicular cells subjected to elevated gonadotrophin apparently do not greatly alter the probability of development of ovarian tumors, nor the time at which they appear. However, the universal finding of tubular adenomas in these C57BL-W^vW^v ovaries, in contrast to the frequent appearance of granulosa-cell tumors and luteomas in other tumorigenic situations, suggests that difference in follicular cells subjected to hormonal imbalance may affect the type of tumor produced. Other factors that may contribute to differences in tumor-type observed here are: extremely early absence of germ cells and estrogen deprivation; possible effects of W^vW^v/ww genic substitution on ovarian stroma or other tissues; and interaction of the reduced germ-cell number with a genetic constitution (C57BL) particularly susceptible to development of tubular adenomas. Evaluation of the effects of some of these variables could be determined by following the history of ovaries in W^vW^v or WW^v females isogenic with other inbred strains or F_1 hybrids.

These histological studies of aging changes in the sterile ovaries of adult W^vW^v females support strongly the contention that the definitive generation of germ cells is derived from the primordial germ cells, and also indicate that lack of definitive germ cells may be a force sufficient for the initiation of a series of events resulting in ovarian tumorigenesis.

References

(*1*) Russell, E. S.: Review of the pleiotropic effects of *W*-series genes on growth and differentiation. *In* Aspects of Synthesis and Order in Growth. Princeton, N. J., Princeton Univ. Press, 1954, pp. 113–126.

(*2*) Russell, E. S., Smith, L. J., and Lawson, F. A.: Implantation of normal blood-forming tissue in radiated genetically anemic hosts. Science 124: 1076–1077, 1956.

(*3*) Silvers, W. K.: Pigment cells: Occurrence in hair follicles. J. Morphol. 99: 41–56, 1956.

(*4*) Mintz, B., and Russell, E. S.: Gene-induced embryological modification of primordial germ cells in the mouse. J. Exper. Zool. 134: 207–238, 1957.

(*5*) Coulombre, J. L., and Russell, E. S.: Analysis of the pleiotropism at the *W*-locus in the mouse: The effects of W and W^v substitution upon postnatal development of germ cells. J. Exper. Zool. 126: 277–296, 1954.

(*6*) Allen, E.: Ovogenesis during sexual maturity. Am. J. Anat. 31: 439–481, 1923.

(*7*) Everett, N. B.: Observational and experimental evidences relating to the origin and differentiation of the definitive germ cells in mice. J. Exper. Zool. 92: 49–91, 1943.

(*8*) Russell, E. S.: Study of life span and pathology tendencies of breeding mice from ten inbred strains. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 245, 1957.

(*9*) Kirschbaum, A.: The role of hormones in cancer: Laboratory animals. Cancer Res. 17: 432–453, 1957.

(*10*) Brambell, F. W. R., Parkes, A. S., and Fielding, D.: Changes in the ovary of the mouse following exposure to X rays. I. Irradiation at three weeks old. Proc. Roy. Soc. London, ser. B. 101: 29–56, 1927.

(*11*) ———: Changes in the ovary of the mouse following exposure to X rays. II. Irradiation at or before birth. Proc. Roy. Soc. London, ser. B. 101: 95–114, 1927.

(*12*) Furth, J., and Butterworth, J. S.: Neoplastic diseases occurring among mice subjected to general irradiation with X rays; ovarian tumors and associated lesions. Am. J. Cancer 28: 66–95, 1936.

(*13*) Bali, T., and Furth, J.: Morphological and biological characteristics of X-ray induced transplantable ovarian tumors. Cancer Res. 9: 449–472, 1949.

(*14*) Furth, J., and Boon, M. C.: Induction of ovarian tumors in mice by X rays. Cancer Res. 7: 241–245, 1947.

(*15*) Lick, L., Kirschbaum, A., and Mixer, H.: Mechanism of induction of ovarian tumors by X rays. Cancer Res. 9: 532–536, 1949.

(*16*) Mühlbock, O.: Hormonal ovarian tumors following roentgen irradiation. Nederl. tijdschr. geneesk. 95: 915–920, 1951.

(*17*) Lorenz, E. O., Jackson, L. O., Heston, W. E., Shimkin, M., Eschenbrenner, A. B., Deringer, M. K., Doriger, J., and Schweisthal, R.: Effects of long-continued total-body gamma irradiation on mice, guinea pigs, and rabbits. III. Effects on life span, weight, blood picture, and carcinogenesis, and the role of intensity of radiation. *In* Biological Effects of External X and Gamma Radiation (Zirkle, Raymond E., ed.). New York, McGraw-Hill, 1954, pp. 24–148.

(*18*) Eschenbrenner, A. B., and Miller, E.: Effects of long-continued total-body gamma irradiation on mice, guinea pigs, and rabbits. V. Pathological observations. *In* Biological Effects of External X and Gamma Radiation (Zirkle, Raymond E., ed.). New York, McGraw-Hill, 1954, pp.169–225.

(*19*) Li, M. H., and Gardner, W. U.: Experimental studies on the pathogenesis and histogenesis of ovarian tumors in mice. Cancer Res. 7: 549–566, 1947.

(*20*) Li, M. H., Gardner, W. U., and Kaplan, H. S.: Effects of X-ray irradiation on the development of ovarian tumors in intrasplenic grafts in castrated mice. J. Nat. Cancer Inst. 8: 91–98, 1947.

(*21*) Li, M. H., and Gardner, W. U.: Further studies on the pathogenesis of ovarian tumors in mice. Cancer Res. 9: 35–41, 1949.

(*22*) Hummel, K. P.: Induced ovarian and adrenal tumors. J. Nat. Cancer Inst. 15: 711–715, 1954.

(*23*) Miller, O. J., and Pfeiffer, C. A.: Demonstration of increased gonadotrophic hormone production in castrated mice with intrasplenic ovarian grafts. Proc. Soc. Exper. Biol. & Med. 75: 178–186, 1950.

(*24*) Mühlbock, O.: Ovarian tumors in mice in parabiotic union. Acta endocrinol. 12: 105–114, 1953.

(*25*) Kaplan, H. S.: Influence of ovarian function on incidence of radiation-induced ovarian tumors in mice. J. Nat. Cancer Inst. 11: 125–132, 1950.

(*26*) Vermande-Van Eck, G. J., and Chang, C. H.: Action of testosterone propionate on the output of pituitary gonadotrophins in mice. Cancer Res. 15: 280–284, 1955.

(*27*) Kirschbaum, A., Liebelt, A. G., and Fletcher, G. H.: Influence of testis on induction of ovarian tumors in mice by X-rays. Proc. Soc. Exper. Biol. & Med. 92: 221–222, 1956.

(*28*) Furth, J.: Discussion of problems related to hormonal factors in initiating and maintaining tumor growth. Cancer Res. 17: 454–464, 1957.

(*29*) Furth, J., and Lorenz, E. O.: Carcinogenesis by ionizing radiations: Endocrine organs. *In* Radiation Biology (Hollaender, A., ed.). New York, McGraw-Hill, 1954, pp. 1167–1172.

(*30*) RUSSELL, E. S.: Recent advances in the study of the pleiotropic effects of genes of the *W*-series. *In* Symposia Genetica 3: 41–45, 1952.

(*31*) RUSSELL, W. L., and RUSSELL, E. S.: Investigation of the sterility-producing action of the W^v gene in the mouse by means of ovarian transplantation. (Abstract.) Genetics 33: 122–123, 1948.

(*32*) BLOOM, W.: The ovary. *In* Histopathology of Irradiation from External and Internal Sources (Bloom, W., ed.). New York, McGraw-Hill, 1948, pp. 598–673.

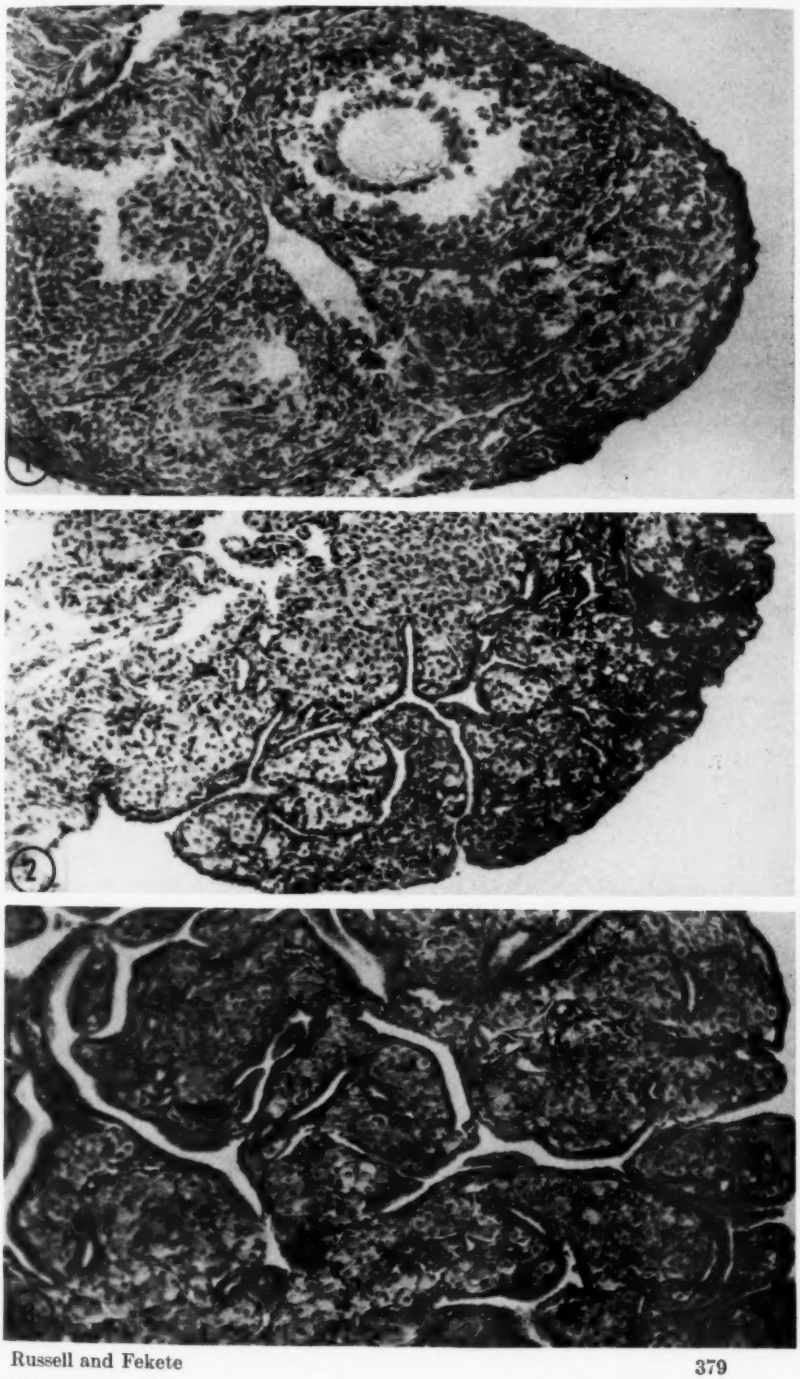
PLATE 43

All sections were stained with hematoxylin and eosin

FIGURE 1.—The ovum in the large follicle shows signs of degeneration. The germinal epithelium is densely crowded together. × 200

FIGURE 2.—The germinal epithelium forms tubular invaginations. × 150

FIGURE 3.—Branching tubules invade the ovary completely. × 150



469463—58——14

PLATE 44

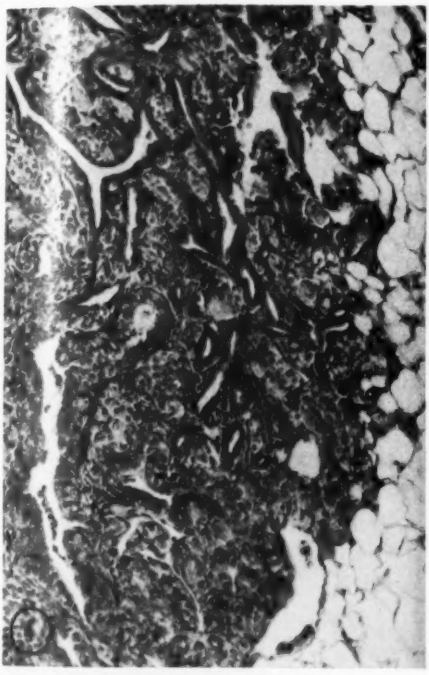
All sections were stained with hematoxylin and eosin

FIGURE 4.—The adipose tissue surrounding the ovary is invaded. × 150

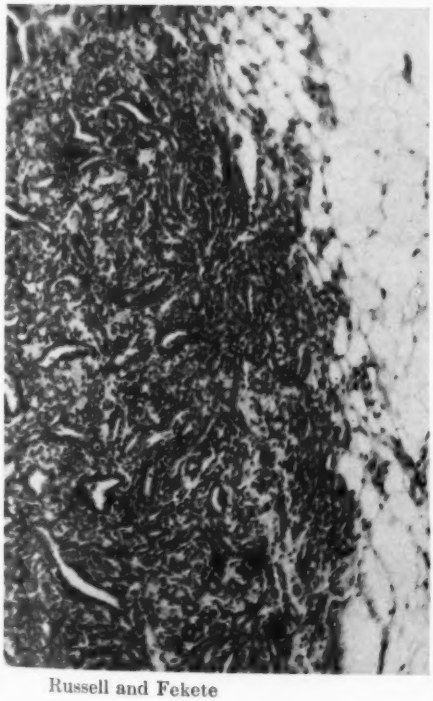
FIGURE 5.—Invasion of the surrounding tissue has occurred at both poles of the ovary. × 30

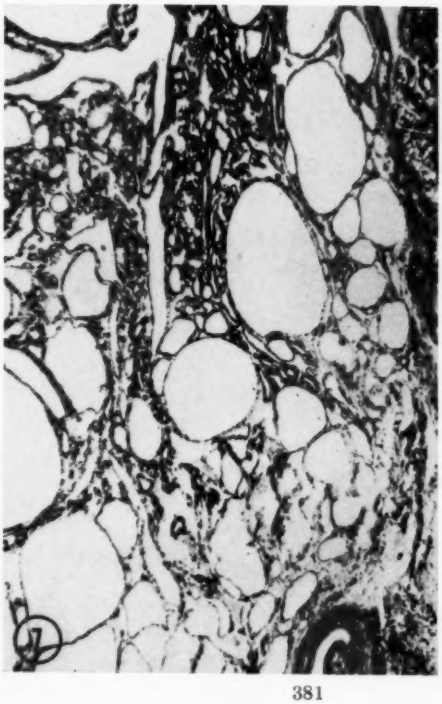
FIGURE 6.—Higher magnification of the lower part of the ovary shown on figure 5. Tubular adenoma invades the adipose tissue. Hypertrophied cells are absent. × 150

Figure 7.—Cystic adenoma of the ovary. × 150



5




7

Evaluation of Data Relating Human Leukemia and Ionizing Radiation [1]

A. W. KIMBALL, *Oak Ridge National Laboratory, Oak Ridge, Tennessee*

Summary

J. Nat. Cancer Inst. 21: 383–391, 1958

The central argument of this paper is a discussion of the statistical reliability of existing data relating human leukemia and ionizing radiation. Conclusions reached by some observers, partly on the basis of statistical techniques having questionable applicability, reveal a confidence in the accuracy of estimated leukemia rates that is difficult to justify. Several sources of error are outlined in detail, and their effect on the use of certain standard statistical methods is illustrated. The conclusion is reached that available data are insufficient to support any conclusion about the shape of the dose-response curve, particularly in the low-dose region.

In the years that have passed since World War II, increasing attention has been given by medical and biological scientists to the effects of ionizing radiation on living organisms. For obvious reasons, the greatest accumulation of knowledge in this field has come from experiments with animals and microorganisms. In spite of the great advances that have been made, we still know relatively little about the effects of radiation on human beings, partly because data of this kind are scarce but mainly because humans are not ordinarily used as experimental subjects when personal risk is involved. Many important questions remain unanswered because scientists are unable to design and execute the necessary experiments. With controlled experimentation virtually eliminated as a source of information, investigators have been forced to rely on retrospective studies. The retrospective study has proved to be one of the most useful tools of medical and public health scientists. Briefly, it consists of selecting from a population individuals having certain characteristics, often some disease, and then conducting a thorough examination of the past experience of each individual in order to determine what factors might be associated with the characteristic. Some of the recent studies on smoking and lung cancer, for example, have been retrospective. Although such studies are

[1] Received for publication March 19, 1958.

469463—58——15

useful when properly evaluated, they can also be misleading when interpreted without regard for pitfalls that are frequently present.

This paper is concerned primarily with the reliability of data relating human leukemia and dose of ionizing radiation. Recently the available data on this subject were assembled and evaluated by E. B. Lewis (*1*). Included in Lewis' survey were individuals from 4 different groups: 1) survivors of the 1945 nuclear explosions in Japan, 2) patients irradiated therapeutically for ankylosing spondylitis, 3) children irradiated therapeutically for thymic enlargement, and 4) radiologists. Strictly speaking, the study of these 4 groups is not retrospective in the sense that individuals were selected because they had leukemia. On the other hand, they were selected retrospectively in the sense that they possessed some special characteristic that led to their irradiation.

It is easy to see how data obtained in this manner might differ from data obtained by first selecting individuals at random from a population and then irradiating them. For example, if one is studying leukemia incidence and it should turn out that the population selected in retrospect is more susceptible to leukemia than the general population, any estimate of incidence obtained from the retrospective study would be biased relative to the general population. The point to be emphasized here is that retrospective studies can be, and frequently are, subject to greater errors than those associated with controlled laboratory experiments.

In some scientific quarters, but by no means in all, Lewis' paper has been interpreted as proof that there is no threshold dose for the induction of leukemia by ionizing radiation. Among the published comments is the editorial entitled "Loaded Dice" by Graham DuShane (*2*), Editor of *Science*, who says in part, "E. B. Lewis (p. 965) shows that there is a direct linear relation between the dose of radiation and the occurrence of leukemia . . .," and, "Thanks to Lewis, it is now possible to calculate—within narrow limits—how many deaths from leukemia will result in any population from any increase in fallout or other source of radiation." Others, while not disputing the widely accepted fact that ionizing radiation does induce leukemia, feel that the question of a threshold dose is far from settled. Without intending to detract from Lewis' otherwise penetrating discussion and summary of existing data on this subject, the present paper purports to show that our knowledge of the correct limits of error for leukemia rates in irradiated human populations is insufficient to support definite conclusions. In this connection, it is relevant that a former member of the Atomic Bomb Casualty Commission, closely connected with the leukemia study in Japan, has expressed similar sentiments (*3*) with respect to the presently available Japanese data.

Basis for Statistical Methods in Research

Statistics is a relatively new discipline among the fields of science, and its use by the research worker has grown rapidly during the past 25 years. This growth has been accompanied by a substantial increase in our knowledge of the mathematical foundations of statistical theory, but in

spite of these advances many kinds of data cannot be examined statistically unless the scientist makes certain assumptions about the conditions under which the data were obtained. Only in rare instances can such assumptions be proved, mainly because experimental subjects seldom behave exactly as if they were black and white balls in an urn. Nevertheless, statistics, when properly applied, has an important role in the evaluation of experimental data, and a careful consideration of the necessary assumptions is essential.

In practice, the statistician is willing to accept some departures from the rigorous framework underlying his methods, but not before he attempts to assess the relative magnitudes of the departures and their possible effect on his ultimate analysis. Generally speaking he prefers to be conservative, and in most cases this means that if he knows that one of two procedures is correct but cannot identify which it is, he will choose the one that yields the larger estimate of error. This assures him that if his analysis leads him to state, "The probability is 0.95 that the interval (A, B) includes the true mean, μ," the correct probability will be equal to or greater than 0.95. Despite his willingness to allow some leeway in the assumptions, every statistician is eventually confronted with data that are subject to a large number of errors about which he has little or no quantitative information. Under these conditions, statistical analysis is of questionable value. There is every indication that the presently available data relating leukemia death rates and radiation dose fall into this category.

The real point at issue is the extent to which Lewis' analysis has advanced our knowledge of the relationship between radiation dose and leukemia incidence, particularly with respect to the existence of a threshold dose. In this connection there is at least a reasonable doubt that the statistical assumptions inherent in his analysis can be justified, and the following sections of this article are intended to establish such doubt. The Japanese data are mentioned most often because they are the only data cited that could conceivably provide direct evidence about low doses, *i.e.*, doses in the range in which a threshold is of most interest.

Recognizable Sources of Error

In the previous section it was stated that the assumptions underlying the use of statistical methods can seldom be proved for any particular experiment. Occasionally it is possible to demonstrate mathematically that certain assumptions, such as homogeneity of errors, are not satisfied, but more often any particular assumption is as hard to disprove as it is to prove. In the majority of cases then, the user of statistical methods must assemble all past and present information that might be pertinent to data of the type in his experiment and that might assist in evaluating the validity of the methods he proposes to use. For mature research workers, most of this information will be the general aggregate of their own past experience. For younger investigators or in unusual experimental situations, it is often not safe to proceed without a careful con-

sideration of the conditions under which the data were obtained, particularly as these conditions relate to the statistical assumptions. The following illustration shows how easily errors in assumptions can arise.

In dominant lethal mutation studies with *Drosophila*, mutation rates are estimated from counts of the numbers of unhatched eggs out of the total number laid. In order to obtain sufficient data, it is usually necessary to make counts for several vials. Because of environmental and other differences, the vial-to-vial variability in percentage unhatched is usually much greater than one would expect for relative frequency data, that is, greater than binomial variability. It is not unusual, however, to find limits of error for data of this type computed on the basis of the binomial distribution, simply because the data take the form of relative frequencies. Such limits, of course, are too narrow, and Cochran (*4*) has given correct methods of analysis for this case. The relevance of this particular illustration should become apparent later.

Let us turn now to the human data relating leukemia incidence and dose of ionizing radiation. The first step in determining what statistical assumptions are reasonable for these data should be a consideration of the errors that may have been involved in their collection. Proper identification of errors is necessary whenever statistical methods are to be used, as the previous example clearly shows. Broadly speaking, errors inherent in experimental data can be classified as gross errors, biases, or random errors. Gross errors result from poor arithmetic, faulty transcription of numbers, etc., and we are not concerned with these in this paper. Biases result from many different causes, the net effect of which is to yield estimates that are consistently too large or too small. Random errors are defined in a succeeding paragraph. Frequently in repeated experiments, errors that ordinarily would be attributed to bias vary from one experiment to another, that is, the bias is not constant. This occurs, for example, when factors capable of affecting the outcome of an experiment, such as age or weight of subjects, are not held at constant levels from one experiment to the next. For convenience, and to distinguish them from truly random errors, these have been called extraneous errors.

Any of the aforementioned types of error may be present in the leukemia data. It would be futile to attempt to cite every possibility, but the specific sources listed below should be sufficient to demonstrate the complexity of the situation for readers unfamiliar with this kind of data.

(*a*) *Dosimetry errors.*—The measurement of radiation dose is not exact even in the best-equipped laboratory. Obviously, in the case of the Japanese exposures, no direct measurements on the ground were possible. Furthermore, the two detonations were qualitatively and quantitatively dissimilar, and the two cities were topographically very different, thus providing quite different shielding effects. Lewis' dose-distance curves were constructed from data presented by Neel and Schull (*5*). These authors give neutron and *gamma* component curves separately for each city. The *gamma* curves are comparable but the neutron component is considerably greater in Hiroshima. The assumption that neutrons are

1.7 times more effective in producing leukemia than *gamma* rays yields remarkably similar dose-distance relationships for the two cities, whereupon the conclusion is made that it is legitimate to pool the leukemia cases from both cities. It is indeed hard to believe that effects in the two cities due to differences in the detonations and differences in shielding have balanced one another in such a way as to yield the curves presented. Certainly Neel and Schull (*5*), who have been very closely associated with this study, give no indication that they were able to reach such a conclusion.

Dosimetry for some of the other sets of leukemia data is possibly somewhat better than the presently available dosimetry in Japan, but these other data present a new problem. Little or nothing is known about the etiology of leukemia. If a threshold dose for the induction of leukemia exists, its magnitude might easily depend on the manner in which radiation is administered, that is, whether in one large dose, in a series of small doses, or chronically. The data include examples of several types of administration, and comparisons among them are certainly not precise enough to support with any confidence the conclusion that acute and chronic radiation exposures yield similar leukemia rates. Thus, if one considers all aspects of the dosimetry problem, it should be clear that the apparent agreement among the calculated leukemia rates per individual per year per rad for the four sets of data may be highly fortuitous. The truncation errors discussed below add further strength to this observation.

(*b*) *Sampling bias.*—In studies on human populations, the question of sampling bias is always foremost. Experts in the field of sample surveys have long been plagued with this problem, several examples of which have occurred in connection with predictions of presidential elections. When sampling bias is present, group differences which appear to be statistically significant with respect to the factor being studied may in fact be due to extraneous factors that were not equally represented in both groups. Efforts are always made to control known sources of bias before samples are taken, but experience shows that these efforts are seldom successful in eliminating all causes of bias. Three sets of the leukemia data deal with very special groups, two that were ill when first irradiated and the third, a group of radiologists. Apparently there is no evidence that would suggest any correlation, other than that due to radiation, between the characteristics of these groups and the incidence of leukemia. On the other hand, with so little really known about leukemia, it would be difficult to state categorically that no such correlations exist. If they do exist, conclusions based on these data could not be applied legitimately to the general population.

Of the four sets of data, the Japanese data are perhaps best from the point of view of sampling bias, in the sense that the exposed population is more representative of the entire population. Here, however, the investigators were forced to rely almost completely on the patient's recollection (several years after exposure) of his position at the time of exposure for the purpose of assigning him to a dose or distance category.

Although great care was exercised to ascertain the accuracy of such recollections, some errors have undoubtedly been made.

(*c*) *Truncation errors.*—From animal studies, it is well known that the distribution of ages at death in irradiated populations is different from that in unirradiated populations. Irradiated populations tend to die earlier, and to some extent certain diseases become relatively more important. Thus, when one compares two such populations with regard to a specific disease incidence before all members of both populations have died, the distributions of ages at death are truncated at different percentage points in the distributions. Clearly, in the Japanese situation, if leukemia victims in the irradiated group tend to die earlier than those in the control group, the incidences that will prevail in these populations after all members have died may be considerably different from the ones Lewis has calculated. In particular, the control group incidence will be relatively greater, and the dose-distance relationship, if linear now, will certainly not be linear when complete data become available. Wald's more recent tabulation (*3*) suggests exactly this, so that truncation errors of this kind are a definite possibility.

(*d*) *Random errors.*—In addition to errors (*a*) to (*c*) and others like them, some or all of which may be negligible in any one experiment, there are the random errors which form the basis of statistical theory. They occur when a population is sampled because the sample is virtually never an exact replica of the population. Only when one's sample includes the whole population do the random errors vanish. In the leukemia data, the random errors are binomial, but the incidences are small enough so that the binomial distribution may be approximated adequately by the Poisson distribution, if desired.

Application of Statistics to the Leukemia Data

With the background provided by the previous sections, it must be clear that before attempting a statistical analysis of the leukemia data, one must decide what assumptions about error are reasonable. Random errors are most certainly present and from statistical theory we know that they would be binomial if the sample sizes were accurately known. In the Japanese data this is not true. The sizes of the exposed populations, *i.e.*, the sample sizes, were estimated by methods described by Neel and Schull (*5*). Thus, appropriate random errors for the Japanese data will be greater than binomial (or Poisson) by an amount related to the precision of the estimated population sizes. Probably more important, however, is the matter of extraneous errors. Neither the existence nor the absence of these errors can be proved, but workers in the field of radiation biology will recognize that some of the errors mentioned in the preceding section and others like them are most certainly present in all of the leukemia data. Our difficulty is that data of this kind are so sparse that we have no way of accurately assessing the magnitude of extraneous errors, and no way of imposing an upper limit on them. Under conditions in which accurate information about errors is lacking, any statis-

tical analysis based on the assumption that only binomial errors are present must be interpreted with reservation.

In view of this, it is appropriate to examine the statistical methods used by Lewis and to see how they are related to the threshold problem. His methods include the construction of confidence intervals based on the Poisson distribution and use of the standard chi-square technique for testing the difference between two binomial populations. The distribution theory that underlies these methods makes no allowance for any errors other than binomial or Poisson errors. To see explicitly how this assumption affects the statistics, consider the chi-square test on page 970 of Lewis' article. Here he is testing the difference between the leukemia incidences among survivors of the Japanese explosions in zone C (p_1) and zone D (p_2), representing average doses of 50 rem and 5 rem, respectively. The formula for χ^2 may be expressed as

$$\chi^2=\left[\frac{p_1-p_2}{\sqrt{p\,(1-p)\left(\frac{1}{N_1}+\frac{1}{N_2}\right)}}\right]^2$$

where N_1 and N_2 are the numbers of exposed individuals and where p is the combined incidence for both groups. It may be seen above that the chi-square test is actually a t test with the variance of (p_1-p_2) assumed to be $p(1-p)\ (N_1^{-1}+N_2^{-1})$; that is, binomial error has been assumed. The data yield a χ^2 of 5.6 which corresponds to a significance level of about 2 percent, as stated. At this point in the discussion it is inferred that because the difference is significant, there can be no threshold dose, at any rate, none above 50 rem. Suppose now that extra-binomial error contributes twice as much to the total variance as does binomial error, not too unusual for some biological data. In this event, the χ^2 would be reduced to 1.9. This corresponds to a significance level of about 24 percent, and one would hesitate to conclude that the observed difference between zones C and D is a real one. The numbers themselves are unimportant, but the possible effect of extra-binomial errors on the tests of significance is of great importance, in view of the conclusions reached by some observ ers. Kendall (*6*) discusses the statistical theory of both extra-binomial and extra-Poisson variation and gives an actual example. He also indicates how difficult it is "to find material compiled outside the laboratory" that conforms to binomial or Poisson distributions.

In various places throughout the article the Poisson distribution has been used to construct confidence limits. It should be pointed out that an error has been made in computing these limits.[2] More important, however, is the fact that the correct Poisson limits are too narrow, perhaps by a large factor, unless all extraneous variation is negligible.

On page 969, 17 observed deaths from leukemia are compared with an "expected" number of 3.4 deaths. Based on the Poisson distribution, a

[2] The limits referred to as 95 percent limits are actually 90 percent limits.

test is made of significance of the difference between the 2 numbers. The test is valid only if there is no extra-Poisson error and if 3.4 is a known constant. In fact, the number 3.4 is not without error and any statistical test would have to take this into account. Apparently the number does represent some sort of arbitrary upper limit on the true expected value, but one cannot make probability statements on this basis.

Conclusions

The most obvious conclusion to be drawn from the foregoing discussion is that statements attributing great accuracy to radiation-induced leukemia rates in human populations are open to serious question, particularly those which suggest a strictly linear dose-response relationship. Lewis did not claim to have established linearity between leukemia incidence and radiation dose, although he implied that linearity is a strong possibility. From the purely methodological point of view, nothing has been done statistically that would either support or disprove the hypothesis of linearity. The linearity hypothesis precludes *per se* the concept of a threshold dose. About the latter, Lewis says, "Thus these data provide no evidence for a threshold dose for the induction of leukemia." and later, "This finding also fails to support the concept of a threshold dose below which leukemia will not develop." One has to admit that Lewis has avoided stating the definite conclusion that there is no threshold dose, although the mode of expression clearly implies this conclusion. The wording is unfortunate because, from a strictly statistical point of view, both statements could equally well have been replaced by similar ones stating the failure of the evidence to support the hypothesis of *no* threshold dose. In other words, the evidence is insufficient to support either hypothesis, and this is about the only conclusion one can safely reach from the data presented. It should also be apparent that the quotation referring to the prediction of radiation-induced leukemia incidence "within narrow limits" has absolutely no basis even by indirect inference from the article. It is important to emphasize this point because other scientists have made statements implying similar beliefs about the precision of the calculations.

Finally, a word of caution is necessary. This article may be interpreted by some readers as representing a point of view opposite to that inferred by Lewis (and adopted more confidently by others), but in reality its only purpose is to emphasize the shaky grounds on which any definite conclusion would be based. Nothing that is said here should be taken as an argument supporting the hypothesis of a threshold dose for the induction of leukemia by ionizing radiation. An attempt to use the same data to establish a threshold would be open to similar criticism. The only sound conclusion to be made is that, in spite of the established fact that ionizing radiation induces leukemia, evidence at the present time is insufficient to justify any hypothesis about the *shape* of the dose-response curve. We can only hope that scientists working on this critical problem will soon provide us with adequate evidence.

References

(1) LEWIS, E. B.: Leukemia and ionizing radiation. Science 125: 965–972, 1957.
(2) DUSHANE, G.: Loaded dice. Science 125: 963, 1957.
(3) WALD, N.: Leukemia in Hiroshima City atomic bomb survivors. Science 127: 699–700, 1958.
(4) COCHRAN, W. G.: Analysis of variance for percentages based on unequal numbers. J. Am. Stat. Assoc. 38: 287–301, 1943.
(5) NEEL, J. V. and SCHULL, W. J.: The Effect of Exposure to the Atomic Bombs on Pregnancy Termination in Hiroshima and Nagasaki. Publ. No. 461, Nat. Acad. Sc.-Nat. Res. Council. Washington, D. C., 1956.
(6) KENDALL, M. G.: The Advanced Theory of Statistics, 2nd ed., revised. London, Charles Griffin & Co., Ltd., vol. 1, 1945, pp. 122–126.

Observations on the Chromosomes of Chinese Hamster Cells in Tissue Culture [1,2]

DENYS K. FORD *and* GEORGE YERGANIAN,[3] *Department of Medicine, University of British Columbia, and British Columbia Medical Research Institute, Vancouver, British Columbia; Children's Cancer Research Foundation, Children's Medical Center, and the Department of Pathology, Harvard Medical School, Boston, Massachusetts*

Summary

J. Nat. Cancer Inst. 21: 393–425, 1958

Serial studies have been made on the chromosomal complement of 4 cell lines derived from the lungs of healthy normal Chinese hamsters and maintained in tissue culture for 3 to 14 months. In the early days of ***in vitro*** life, the cell lines were characterized by a diploid chromosomal complement, but metaphases with an approximately tetraploid number soon appeared. When diploid cells became a minority, there were usually significant numbers of approximately triploid and more-than-tetraploid metaphases. Detailed chromosomal analysis revealed abnormalities in cells which, to superficial examination, would have been classed as diploid. Particular patterns of extra chromosomes, absent chromosomes, functioning half chromosomes, and aberrant chromosomes characterized 2 of the cell lines and also a methylcholanthrene-treated subline of 1 of them.

Cultures derived from normal rat and mouse tissues may undergo spontaneous transformations *in vitro* to malignant cells capable of causing tumors at the site of reimplantation into animals of the strain originating the cultures (*1*, *2*). These initial observations have been extended to cell clones (*3*), and the concept that cell transformations may be of frequent occurrence has been suggested (*4*). That the findings are applicable also to cells of human origin is indicated by reports (*5–7*) which note mitotic and chromosomal aberrations in the transformed cells. Detailed studies of the chromosomes of several established human cell strains have been made (*8*), and the presence of abnormal numbers has been used as evidence that a malignant change may have occurred in the cultured cells. The study of chromosomes in tissue cultures has been continued since 1913 (*9*), and was the subject of repeated investigation by Lewis and Lewis (*10–15*). Nevertheless, no detailed serial observations on the chromosome

[1] Received for publication February 19, 1958.

[2] Supported by the National Cancer Institute of Canada: Project No. 178.

[3] The authors wish to thank Dr. Kenneth A. Evelyn, Director of the British Columbia Medical Research Institute, for much helpful advice, and Mrs. Lee Harper, for technical assistance.

complement of cells derived from normal tissues have been reported, and correlations between changes in the behavior of cultivated cells and their chromosomal patterns are not understood.

The successful laboratory breeding of the Chinese hamster, *Cricetulus griseus*, (*16*) has made available a mammal whose cells have only 11 relatively distinct pairs of chromosomes, a feature that makes this animal of particular value for cytogenetic research (*17*). Therefore tissue from the Chinese hamster was selected for studying chromosomes of cells cultivated *in vitro* for prolonged periods. The plan of the investigation was to initiate a number of cell lines from normal tissues and to observe these cells at intervals with chromosome counts, phase-contrast microscopy of the living cultures, and May-Grünwald-Giemsa-stained preparations of fixed specimens. It was hoped that correlations could be made between changes in cell behavior and the chromosome constitution of the cells.

Materials and Methods

Pyrex or Kimble glassware was used throughout. Cultures were grown in milk-dilution bottles and modified T-15 flasks, with white rubber stoppers, or in screw-cap culture tubes, with and without 22 × 7 mm. Corning coverslips. The Calgon-metasilicate glass-washing routine suggested by the Tissue Culture Association (*18*) was adopted. To Connaught Laboratories' medium 1066 was added 100 units of penicillin and 100 μg. of streptomycin per ml. This synthetic medium was then supplemented with 20 percent Seitz-filtered calf serum, obtained from a local abattoir, and also with 0.5 percent lactalbumin hydrolysate that was sterilized by autoclaving. All media were gassed with 5 percent CO_2 in air when indicated, to adjust the *p*H 7.2 to 7.5. Trypsin was obtained from Difco as Bacto-Trypsin in sterile ampoules, and the Hanks' solution was prepared in the laboratory and sterilized by autoclaving.

The lungs of young adult male Chinese hamsters, from the Boston colony, were removed under anesthesia, washed twice in Hanks' solution, minced with scissors, and washed again twice in Hanks' solution. Three to 5 successive 10 ml. portions of 0.25 percent trypsin in Hanks' solution, lacking calcium and magnesium, were allowed to digest the minced lung tissue at 37° C. and *p*H 7.4; after each 30- to 45-minute trypsinization, including vigorous pipetting, the cell suspension was removed to a flask, and stored in the refrigerator while the new trypsin was added. The accumulated cell suspension was poured through a double layer of gauze to filter off the larger tissue fragments, and then centrifuged at 1000 r.p.m. for 10 minutes. After the supernatant trypsin was discarded, the cell deposit was resuspended in 7 ml. of medium and pipetted into a milk-dilution bottle. Culture media were changed at 2- to 4-day intervals, and subculture by standard trypsinization procedures (*18*) with 0.25 percent trypsin was carried out whenever cell growth was adequate—a cell concentration of approximately 250,000 cells per ml. being found suitable. All stock cultures of each cell strain were grown in duplicate bottles in each of 2 incubators, and their respective media were kept

separate to reduce the chance of accidents destroying all of any cell line; these duplicate cultures were called sublines 1 and 2 of their respective cell strains.

Preparations for chromosome staining were obtained from 3-day-old cultures 24 to 30 hours following a medium change. After the medium was discarded, 0.25 percent trypsin was added for 10 minutes and the resulting cell suspension centrifuged for 5 minutes. The trypsin was then poured off and the cell deposit suspended in a hypotonic solution (Hanks' without sodium chloride 70%, isotonic Hanks' 30%) for 5 to 10 minutes. Following 5-minute centrifugation and removal of the hypotonic solution, the cell deposit was fixed in acetic-alcohol (1:3) and stored in screw-cap vials labeled with the cell strain and date of fixation. Later in the study, the cells were pretreated with colchicine by adding 0.2 ml. of a 1-in-a-100,000 colchicine solution to the 2 ml. of medium in the culture tubes; after 2 hours' incubation the medium was poured off and the hypotonic Hanks' solution was added for 10 minutes before being replaced by the acetic-alcohol mixture.

Squash preparations were made by placing 1 drop of the fixed cells on a slide, and then adding 1 drop of freshly filtered acetic-orcein (1% synthetic orcein in 45% acetic acid). A coverslip was then placed on the combined drops, the excess stain blotted off, and the coverslip squashed with the thumb, after which the edges of the coverslip were ringed with a gelatin-acetic acid mixture and later with balsam. These orcein preparations were scanned with a $\times 20$ objective and the metaphases were studied with the oil-immersion lens. Initially, 25 countable metaphases from each specimen were drawn with a camera-lucida attachment and all the metaphases seen during this procedure were recorded to find the proportion of the metaphase population that was countable; an error of 5 to 10 percent in chromosome number of the cells selected for drawing was permitted so that a reasonably high percentage of metaphases could be drawn, and under these circumstances usually about 30 to 50 percent of the metaphases were acceptable for counting. Later, when the individual chromosomes of the metaphase plates were being closely studied, a high degree of selection was necessary but the counts were completely accurate. In addition, other preparations of each specimen were scanned and estimates made of the chromosome number of 50 to 100 consecutive metaphases in order to determine the distribution of metaphases between various chromosome-number groups. These estimates were of sufficient accuracy to divide the metaphase population into an approximately "diploid" group with 20 to 25 chromosomes, a "triploid and tetraploid" group with between 30 to 50 chromosomes, and a "more-than-tetraploid" group with over 50 chromosomes. Selected metaphases were photographed and phase-contrast optics were used throughout.

Cell morphology in the living cultures was observed in T-15 flasks with a $\times 10$ objective and phase-contrast optics; selected cultures were photographed. In addition, methyl alcohol-fixed coverslip cultures were stained at intervals by the May-Grünwald-Giemsa method. A Leica

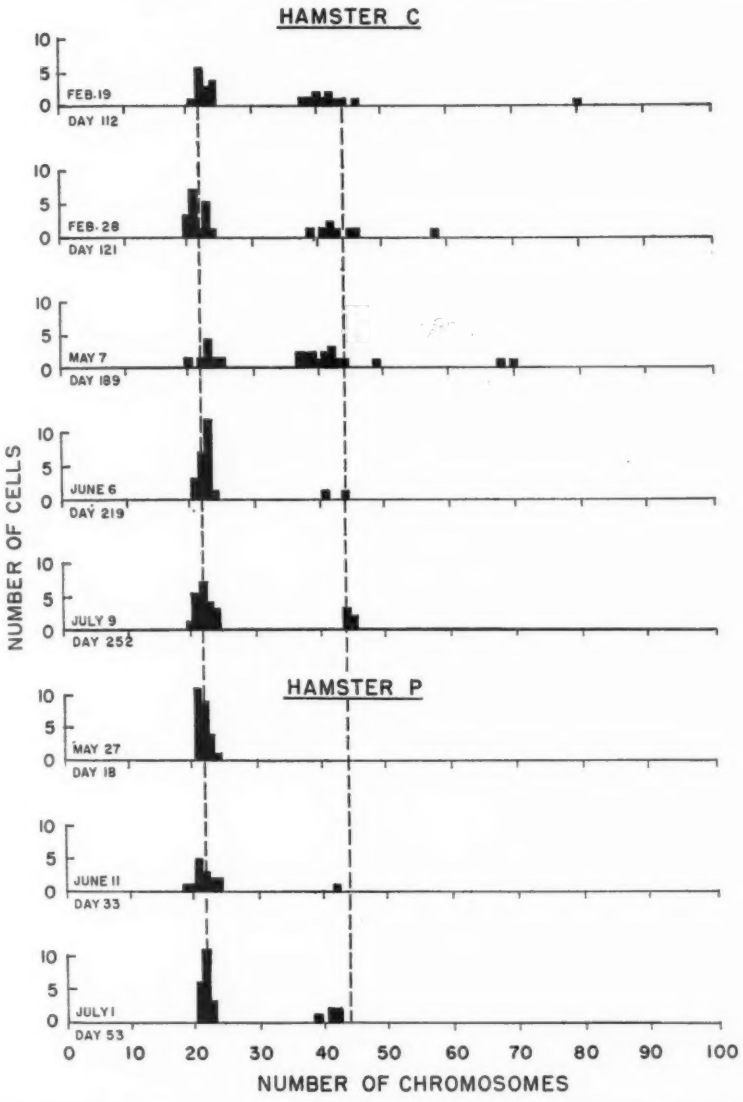
35 mm. camera body and Leitz camera attachment were incorporated with the Reichert Zetopan microscope for photography; Kodak Panatomic × film, an exposure time of 2 seconds with a green filter, and Microdol developer were used.

Results

The first 3 lung cultures A, B, and C, from different animals, initiated by the techniques mentioned at the end of October, 1956, proliferated well and cell lines A and C have been continued in culture since; line B ceased to grow and was discarded after 10 weeks. During November and December, 1956, 7 cultures were prepared from trypsinized testes, which were removed under anesthesia so that the animals could live and be available for reimplantation experiments with their autologous cultures at a later date. However, in spite of early cell multiplication, all these cultures eventually degenerated after 2 to 5 weeks, and the investigation was therefore limited to lung strains. In April and May, 1957, 6 attempts to initiate new lung cultures by identical techniques gave only one active cell strain, line P, and again in August and September, 1957, a further cell line, V, was started from another 7 attempts.

The cells obtained by these methods have been considered as fibroblasts, and typically have appeared spindle-shaped, though on occasion, and particularly in lines A and P, the cells have been more flattened and epithelium-like. These appearances are illustrated by the phase-contrast photographs of living cultures in figures 1 and 2. May-Grünwald-Giemsa-stained coverslip preparations have shown considerable variation in size and shape of both cells and nuclei, the latter containing 1 to 5 nucleoli; a moderate number of the cells of all cultures have been multinucleate. With one exception, to be discussed later, the cellular morphology of these cultures, whether seen in stained or live preparations, was too variable to permit conclusions to be drawn. Similarly, the growth rate of the cell lines varied greatly without evident cause, but no regular quantitative measurements of growth rate were obtained.

Histograms of chromosome counts from successive cultures of cell lines C and P are given in text-figure 1, which shows that the chromosome numbers lay predominantly in the diploid and tetraploid regions. In table 1 the metaphase populations of lines C, P, and V have been divided on the basis of chromosome estimates of consecutive cells into "diploid," "triploid and tetraploid," and "more-than-tetraploid" groups. The text-figure and table together outline the sequence of events for these three cell lines. The V culture was observed first at 11 days when 90 percent of the cells were diploid, and at 53 days, 86 percent were still diploid (table 1). The P culture was first examined at 18 days when chromosome counts of 25 cells showed all to be diploid (text-fig. 1); although no chromosome estimates were available at this time, 68 percent of the metaphases of the specimen were acceptable for drawing. At 33 days 84 percent of the cells were diploid and thereafter up to the 175th day about three fourths of the cells were diploid (table 1). A few "more-than-tetraploid" cells appeared after



TEXT-FIGURE 1.—Chromosome counts of metaphases from successive cultures of cell lines C and P. Due to metaphase selection for counting, the relative numbers of metaphases with different chromosome counts are not obtainable from the text-figure but only from table 1.

100 days in culture, but these predominantly octaploid cells remained infrequent.

Line C cells were first studied at the 112th day when about 40 percent of the metaphases were diploid; a similar number were in the tetraploid

TABLE 1.—*Chromosome estimates of cell lines C, P, and V*

Cell line	Date	After initiation of culture (*days*)	Metaphases observed (*No.*)	Metaphases "diploid" (*percent*)	Metaphases "triploid or tetraploid" (*percent*)	Metaphases "more-than-tetraploid" (*percent*)	Metaphases "not estimable" (*percent*)
C	Feb. 19	112	75	41	43	11	5
	Feb. 28	121	50	44	46	6	4
	May 7	189	50	40	48	6	6
	June 6	219	50	72	28	0	0
	July 9	252	50	92	6	0	2
	Aug. 9	283	100	31	45*	18	6
	Aug. 20	294	100	48	39*	8	5
	Sept. 28	333	100	51	41*	6	2
	Oct. 24	359	100	63	33*	1	3
P	May 27	18	—	—	—	—	—
	June 11	33	25	84	16	0	0
	July 1	53	50	68	28	0	4
	July 29	81	50	66	34	0	0
	Aug. 23	106	50	70	22	2	6
	Sept. 12	126	100	56	34	6	4
	Sept. 26	140	100	74	24	2	0
	Oct. 31	175	100	83	13	4	0
V	Sept. 23	11	100	90	3	0	7
	Nov. 4	53	100	86	7	0	7

*On these 4 occasions, 13, 18, 5, and 12 of the cells, respectively, had a chromosome number between 30-39.

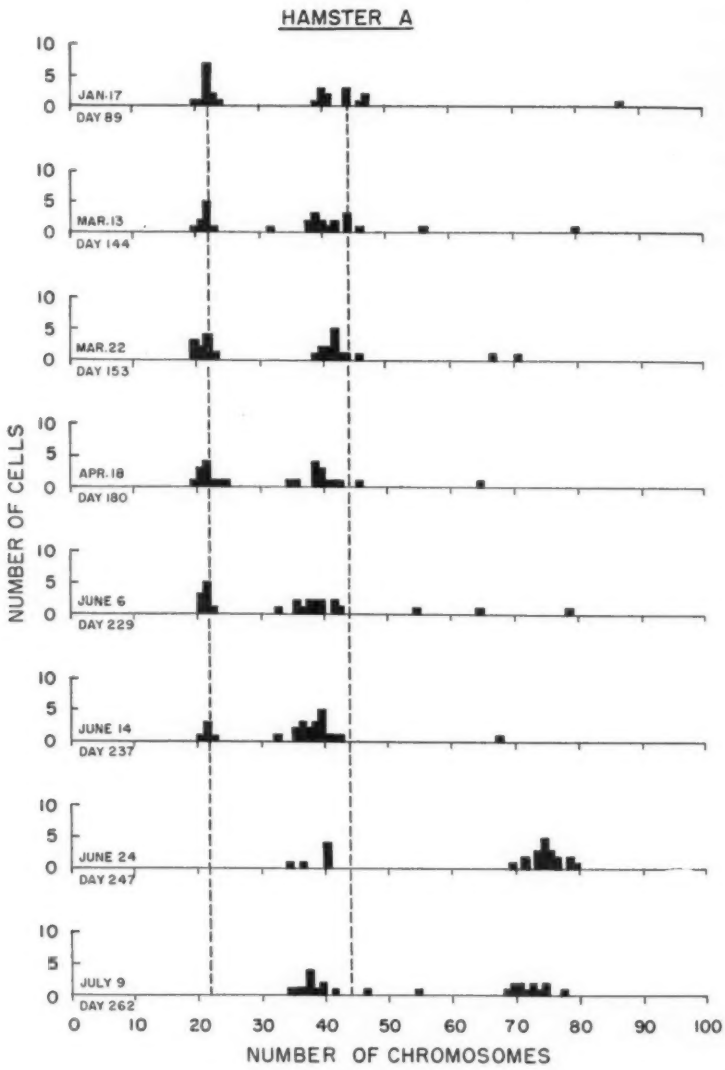
region, and a few were hypertetraploid (table 1). These proportions remained the same for the next 11 weeks. Text-figure 1 shows that some of the hyperploid cells were tetraploids and some hypotetraploid, the presence of the latter being most evident at 189 days when 6 out of 25 cells had 37 to 39 chromosomes. Subsequently, at 219 and 252 days chromosome estimates of fixed-cell specimens from C cultures appeared more "normal," and 70 and 90 percent of the metaphases, respectively, were found to be diploid. It seemed that with this reversal to diploidy the hyperploid cells became predominantly tetraploid, and on July 9th, the 252nd day, the

5 counted hyperploids had 44 to 45 chromosomes. The C strain grew very slowly during March and April so that it was not possible to obtain specimens for chromosome study and it was feared the culture might be lost, but during June and July when predominantly diploid, it proliferated rapidly. In mid-August the #2 subline of the C cultures spontaneously ceased to multiply and gradually deteriorated to a complete loss of the subline over the following 2 months. The #1 subline continued to grow well till the last week of August when the cultures became inactive for a month before spontaneously reviving and returning to the former state of continuous proliferation. When still active in August, the number of diploid cells fell with a corresponding increase in hyperploid cells, and thereafter about half to two thirds of the cells were diploid; recently metaphases with chromosome counts between 30 and 39 have formed an appreciable proportion of those with increased chromosome numbers. At no time has the cellular morphology of the C cultures varied significantly, except that inactive cultures have been characterized by moderately rounded and granular cells.

Line A cultures differed markedly in behavior from line C. When observations were started at 89 days, about a quarter of the cells were diploid and the remainder hyperploid (table 2), and approximately this pattern was maintained in the next 2 observations. On the 180th day only 16 percent of the metaphase population were diploid and, in contrast, 24 percent were in the octaploid region; at the same time, 6 out of 25 counted metaphases had 35 to 39 chromosomes (text-fig. 2). Until the 234th day the A cultures were of fibroblastic appearance but between the 234th and 236th days a very definite change was noted in the cellular morphology of the A cultures. This difference appeared simultaneously in the duplicate stock A bottles of each incubator, the cells of these 2

TABLE 2.—*Chromosome estimates of cell line A*

Date	After initiation of culture (*days*)	Metaphases observed (*No.*)	Metaphases "diploid" (*percent*)	Metaphases "triploid or tetraploid" (*percent*)	Metaphases "more-than-tetraploid" (*percent*)		Metaphases "not estimable" (*percent*)
					"Normal" chromosomes	? Human chromosomes	
Jan. 17	89	100	23	67	9		1
Mar. 13	144	100	30	59	4		7
Mar. 22	153	100	29	58	10		3
Apr. 18	180	100	16	54	24		6
June 6	229	50	14	66	8	10	2
June 14	237	100	4	18	1	72	5
June 24	247	100	0	5	0	94	1
July 9	262	100	4	21	3	63	9
Aug. 8	292	100	0	0	0	100	0



TEXT-FIGURE 2.—Chromosome counts of metaphases from successive cultures of cell line A. Due to metaphase selection for counting, the relative numbers of metaphases with different chromosome counts are not obtainable from the text-figure but only from table 2. The metaphases with 70 to 80 chromosomes on the 247th and 262nd days were believed subsequently to be derived from a contaminating human cell strain.

stocks having had no known contact with each other for over 3 months; but in contrast, 1 bottle of A cells, which a week before had been cultured in a 1-in-10-million dilution of colchicine, underwent this change more

slowly and several weeks later. Most of the new cells seemed smaller and more uniform in size and were either rounded or spindle-shaped as compared to the previous larger and flattened A cells. The round cells appeared to fall readily off the glass in spite of seeming healthy and active in proliferation. The growth rate of the cultures increased but the significance of this was obscure as the C cultures that showed normal cellular morphology also began to grow faster at this time.

At first it was thought that a cell alteration might have occurred in the A cultures and the appearance of a new type of metaphase in the chromosome preparations of these cultures was considered supporting evidence. The new class had 70 to 80 chromosomes (text-fig. 2) of a smaller size than those normally found in Chinese hamster cells. Four days after the change was first noted, chromosome estimates (table 2) indicated that 72 percent of the metaphases were of this changed type; however, when the preparation taken on the 229th day was reviewed, 4 days before the change was recognized, 10 percent of the cells were seen to have the same characteristics. Subsequent close study with the aid of colchicine pretreatment of the chromosomes of these changed A cells revealed that they were indistinguishable in appearance from the chromosomes of the 3 human cell lines in the laboratory at the time (figs. 3, 4, and 5). Chromosome counts of the HeLa and KB human cell lines showed them to have 73 to 74 and 75 to 76 chromosomes, respectively, and the mean cell count for the changed A line was 75; the Chang conjunctival cell strain had 82 to 84 chromosomes. Unsuccessful attempts were made with human anti-A and anti-B blood-typing sera and also with a commercially prepared anti-human-protein serum to relate these A cultures serologically to the human cell lines. Subsequently, antisera against the C and P normal Chinese hamster cells, against the changed A cells, and against a combination of the 3 human cell strains were prepared in 3 rabbits. Each rabbit received 3 intravenous injections of 10 to 20 million cells at 21-day intervals, and the sera obtained 10 days after the 3rd injection were tested for cytotoxic activity on tube cultures of both A and P cell strains, the latter being of indisputable Chinese hamster origin. The changed A cultures were very markedly damaged within 12 hours by the anti-A and anti-human sera in a dilution of 1:10, but were not affected by the anti-Chinese hamster serum. In contrast, the P cultures showed marked cytotoxic damage from the anti-Chinese hamster serum. Thus, the supposition that the changed A cultures represented an overgrowth of the original Chinese hamster cell line by a contaminating human cell line would seem to have been proved, though it was not possible to say how or when this contamination could have occurred.

Before suspicion was aroused as to the true origin of the changed A cells, live representatives of the normal C and the changed A strains were transferred to Boston where they were continued in culture so that implantation experiments could be performed to demonstrate tumor-producing properties on the part of the cells. The cheek pouches of Chinese hamsters not pretreated with cortisone or irradiation were injected with

inocula of the respective cultures, which were not isologous with the recipient animals. Fourteen cheek pouches were implanted with line C cells with no visible host reactions during the following 4 to 6 days when such reactions were expected to take place with noncompatible tissues. Sixteen cheek pouches in 8 animals were implanted with the A line and subsequently small nodules developed at the site of implantation. A photograph of a section from a nodule is shown in figure 6, and the proliferative nature of the lesion is indicated by the number of mitoses. Over a 60-day period a larger nodule arose in the other cheek pouch of the same animal. Unfortunately, the fact that the A cells are capable of inducing tumors at the implantation site in nonirradiated, uncortisonized Chinese hamsters does not prove their origin to be from this species. Foley and Handler (*19*) have demonstrated that HeLa, KB, and other human cell lines may give rise to cheek-pouch tumors in unconditioned Syrian hamsters, and thus in the cheek pouch these cultured cells are capable of overcoming the immunologic barriers of heterologous hosts.

The observations already described on the number of chromosomes in the metaphases of these 4 cell lines indicate that the pattern in cultured strains is not constant. From the findings in P and V it appears that the cultures are predominantly diploid at the start, but that tetraploid cells soon appear. In cultures established for several months, the degree of diploidy is variable from a low of 16 to a high of 92 percent; thus line C initially seemed stable with 40 percent diploid but subsequently the number rose to 92 percent, fell to 30 to 40 percent, and more recently has been 50 to 60 percent. The rate of growth of the cultures could not be correlated closely with diploidy, though this was a marked feature while the C cultures were growing fast in June and July. When the cell lines were predominantly diploid, the hyperploid cells had chromosome numbers close to 44, but when diploids were infrequent, an appreciable proportion of approximately triploid and hypertetraploid cells were usually found.

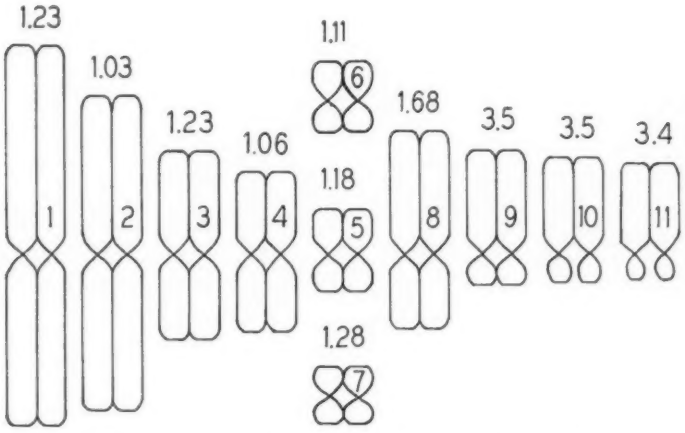
After a background of information on chromosome number was obtained, the individual chromosomes and their distribution were closely studied in diploid metaphases of the cultures. Text-figure 3 is a diagrammatic representation of the 11 pairs of chromosomes in normal Chinese hamster cells, the chromosomes being characterized by their size and the position of the centromere. The ease with which pairs can be recognized is variable and the greatest difficulty arises in differentiating pairs 6 and 7, and pairs 10 and 11; colchicine pretreatment is essential for this work. As an aid to the recognition of the individual chromosomes in the photographs of diploid metaphases (figs. 7 to 26), corresponding diagrams of the metaphases have been included; the diagrams of the chromosomes have been drawn solely to assist in the recognition of the chromosomes in the photographs and otherwise have no morphologic significance. Table 3 shows the incidence of particular characteristics in metaphases of cell lines C and P, while table 4 gives

TABLE 3.—*Incidence of particular characteristics in "diploid" metaphases of cell lines C and P, 12 and 6 months, respectively, after initiation of the cultures*

Cell line	Chromosome count*	Number of metaphases	Number of metaphases with particular characteristics							
			Classical diploid	Normal complement of #3, 4, and 8	An absent #3, 4, or 8	Two atypical telocentrics (*T*)	One atypical telocentric (*T*)	Extra #6 or 7	Extra #10 or 11	Miscellaneous†
C	20 + T	1		1			1			1
	21	1		1						1
	21 + T	3		1	2		3	1		1
	22	2	1	1	1	1				
	22 + T	2		1	1		2	1	1	1
	23	9		7	2	7		3	2	1
	23 + T	13		13			13	12		2
	24	17		5	12	13		17	15	2
	25 to 26	2		2			1	2	2	2
	Total	50	1	32	18	21	20	36	20	11
	Percent	.....	*2*	*64*	*36*	*42*	*40*	*72*	*40*	*22*
P	20 + T	1			1		1			1
	21	2		1	1					1
	21 + T	1			1		1			
	22	24	11	13	11	7		2	4	4
	22 + T	12			12		12		12	
	23	7		3	4	4		2	5	1
	23 + T	1			1		1		1	1
	24	2		2		1			2	1
	Total	50	11	19	31	12	15	4	24	9
	Percent	.....	*22*	*38*	*62*	*24*	*30*	*8*	*48*	*18*

*Two atypical telocentrics associated with an absence of a #3, 4, or 8 are presumed, for the purposes of these counts, to have arisen from centric breakage of the absent chromosome and are therefore counted as 1 chromosome.

†Miscellaneous abnormalities not included in the other columns and occurring sporadically so that their detailed analysis is being deferred until additional data are available.



TEXT-FIGURE 3.—Diagram of the 11 chromosome pairs in normal Chinese hamster metaphases: the figures above the chromosomes give the ratio between the lengths of the 2 arms of each chromosome.

the common patterns of chromosome distribution and aberrations that were found in cell lines C, P, and V. Fifty diploid metaphases were examined from each cell line.

Eighty-two percent of 50 V metaphases (table 4) studied about 2 months after initiation of the culture were classically diploid as illustrated in figures 7 to 10. However, the detailed examination of metaphases in both the C and P cultures during October and November, 1957, 12 and 6 months, respectively, after the cultures were initiated, revealed that definite chromosomal abnormalities were present in seemingly diploid cells.

In addition to other features, both of these cell lines showed new chromosome forms considered to be functioning half chromosomes arising from centromeric fracture of a medium-sized metacentric chromosome.

TABLE 4.—*Incidence of common chromosomal patterns in "diploid" metaphases of cell lines C, P, and V, 12, 6, and 2 months, respectively, after initiation of the cultures*

Cell line	Cells with a particular pattern (*percent*)	Chromosome count		Absent or extra chromosomes
		Normal chromosomes	Atypical telocentrics	
C	24	23	2	Absent #3, 4, or 8 Extra #6 or 7 Extra #10 or 11
	22	23	1	Extra #6 or 7
P	22	22		Classical diploid
	24	22	1	Absent #3, 4, or 8 Extra body resembling a #10 or 11
	14	21	2	Absent #3, 4, or 8
V	82	22		Classical diploid

These half chromosomes, hereafter called atypical telocentrics and labeled "T" in the diagrams, were probably derived from fracture of one of the pair of #3 chromosomes, because when 2 atypical telocentrics were found only one #3 chromosome was present. Although in many cases it was difficult to distinguish between the #3, 4, and 8 chromosomes of these C and P cultures, and centric fracture of a #4 or 8 could in these cases have originated the atypical telocentric, the impression remained that one of the #3's had in fact originated them. On occasions when an atypical telocentric was found in the presence of a full complement of #3, 4, and 8 chromosomes, its origin could not readily be explained.

Table 4 shows that the commonest metaphase pattern in the C line had a chromosome complement of 23 + 2 atypical telocentrics and that this was found in 24 percent of the metaphases. In addition to the telocentrics, probably arising from the absent #3 chromosome as already stated, the metaphases had extra #6 or 7 and extra #10 or 11 chromosomes. Figures 11 to 14 show photographs and numbered diagrams of 2 examples of this metaphase type. Although each medium-sized metacentric chromosome in the diagrams has been given a number, the only certainty is that one of the #3, 4, or 8 chromosomes is missing. In the photographs the 2 telocentrics are evident in each metaphase and are distinguishable from the subterminal chromosomes; 5 small metacentric chromosomes #6 and 7, and 5 smaller subterminal chromosomes #10 and 11 are also in each metaphase.

Of approximately the same frequency, 22 percent in table 4, was a slightly different metaphase with a count of 23 + 1 atypical telocentric. This complement was formed of the classical number and distribution, with the superimposition of an extra #6 or 7 and the telocentric, the origin of which was not evident because no chromosome was absent or fragmented. Photographs and diagrams of this metaphase type are shown in figures 15 to 18.

Table 3 shows that 72 percent of the "diploid" C metaphases had an extra #6 or 7 chromosome, and that about 40 percent, respectively, had an absent metacentric #3, 4, or 8, 2 telocentrics, 1 telocentric, and an extra subterminal #10 or 11. Only 1 classical diploid cell was seen in the 50 metaphases examined and in contrast 86 percent of the metaphases were hyperdiploid in that they had more than 22 chromosomes.

The "diploid" metaphases of line P demonstrated other abnormalities, and 22 percent of the cells were still classical diploids (table 4). Twenty-four percent had a normal complement with the exception that 1 of the metacentric #3, 4, or 8 chromosomes was absent and replaced by an atypical telocentric and another body that could not usually be distinguished from a subterminal chromosome, #10 or 11; it was probably the other half of the absent metacentric rather than an extra #10 or 11. This opinion was strengthened by the presence of another 14 percent of cells in which the absent metacentric was replaced by 2 atypical telecentrics. Photographs and diagrams (figs. 19 to 22) of 2 examples of the metaphases with 1 telocentric and 1 extra #10 or 11 show how the former

differs from the group of subterminal chromosomes which appear to have an additional member. In contrast to the C line, only a few cells had extra #6 or 7 chromosomes, and the degree of hyperdiploidy was less in these P cultures.

During June, 1957, 1 flask of the C line was exposed for 28 days to methylcholanthrene, 10 μg. per ml. of medium. The methylcholanthrene was partially in suspension, and the bottle containing the medium and 12 glass beads was shaken 500 times before medium was transferred to the cultures. All the glassware and rubber stoppers contacting methylcholanthrene, and also the treated cells for the following 4 months, were destroyed, but the culture was otherwise handled in the same manner as the stock cell strains. Successive estimates of the chromosome number of this treated culture are given in table 5; a fall in diploids and a marked rise in approximate triploids are the outstanding features. Detailed chromosome analysis of 25 "diploid" metaphases 4 months after treatment revealed the surprisingly uniform pattern shown in table 6. The remarkable characteristic was the presence of an unusually large subterminal chromosome which was found in 23 out of 25 metaphases. In addition, the great majority of metaphases had counts of 21, an absence of a metacentric #3, 4, or 8 chromosome, an absence of a small metacentric #6 or 7, and an abnormally short arm to one of the #1 chromosomes. A combination of all of these features was found in 16 out of 25 metaphases. Figures 23 to 26 illustrate the appearance of these characteristic hypodiploid metaphases.

TABLE 5.—*Chromosome estimates of the methylcholanthrene-treated subline of cell line C*

Date 1957	After onset of treatment (*days*)	Cells estimated (*No.*)	"Diploid" (*percent*)	"Triploid" (*percent*)	"Tetraploid" (*percent*)	"More-than-tetraploid" (*percent*)	"Not estimable" (*percent*)
Aug. 1	62	100	44	23	10	17	6
Oct. 10	132	100	47	16	31	2	4
Oct. 24	146	100	38	12	27	19	4
Nov. 15	168	100	28	56	3	10	3

TABLE 6.—*Abnormalities encountered in "diploid" metaphases of the methylcholanthrene-treated subline of cell line C*

Cells	Cells analyzed	Count 21	Large subterminal (LS) chromosome	Absent #3, 4, or 8	Absent #6 or 7	Short-arm #1	Characteristic pattern (*Count of 21; absent #3, 4, or 8; absent #6 or 7; short-arm #1; LS present*)
Number	25	19	23	22	22	18	16
Percent	100	76	92	88	88	72	64

This detailed study of the chromosomal complement of "diploid" metaphases has revealed certain events common to more than 1 cell line. Thus, extra chromosomes of the #6 and 7, and #10 and 11 groups, causing slight hyperdiploidy, and the presence of 1 or 2 atypical telocentrics, derived sometimes from centromeric breakage of a metacentric, were features in both the C and P cell lines after 12 and 6 months of *in vitro* culture, respectively. In contrast, the pattern in the methylcholanthrene-treated C subline was quite distinct and clearly marked the cells of this culture from the others. The analysis of metaphases containing grossly increased numbers of chromosomes has not yet been completed, but figures 27 and 28 show atypical telocentrics in approximately tetraploid C and P metaphases, while figure 29 illustrates the duplication of the large subterminal chromosome in an approximately triploid metaphase from the methylcholanthrene-treated C subline. In figure 30 endomitosis in a hyperdiploid C metaphase has occurred, and the doubling of an atypical telocentric is evident.

Discussion

Before the significance of chromosomal abnormalities in cultured malignant cell strains can be evaluated, the behavior of "normal" cells from the initiation of their *in vitro* life must be understood. It is with this objective that these experiments have been carried out, but many cell strains will have to be observed for long periods before characteristic patterns are demonstrable. The observations here described form part of a long-term program of chromosomal study in cultured Chinese hamster cell strains which are being implanted intermittently into homologous hosts to demonstrate neoplastic transformations. The significance of cheek-pouch implantations with homologous cells from partially inbred animals will have to be evaluated, but the findings of Foley and Handler, already discussed, would suggest that immunologic identity may not be required for the growth of cultured tumor cells implanted into hamster cheek pouches. The further investigation of chromosome distribution and morphology with the extension of the observations to hyperploid cells is being continued.

As soon as it seemed evident that the A line was overgrown by a more active contaminating human cell strain, the technical procedures of the laboratory were rigorously overhauled to render impossible the further accidental transfer of cells from one cell line to another. The contamination of the A strain is the only example of such an occurrence in these Chinese hamster cultures because in recent months cell lines C, P, and V have each maintained their own particular chromosomal complement of the distinctive Chinese hamster pattern.

References

(*1*) Gey, G. O.: Cytological and cultural observations on transplantable rat sarcomata produced by the inoculation of altered normal cells maintained in continuous culture. (Abstract.) Cancer Res. 1: 737, 1941.

(2) EARLE, W. R., and NETTLESHIP, A.: Production of malignancy *in vitro*. V. Results of injections of cultures into mice. J. Nat. Cancer Inst. 4: 213–227, 1943.

(3) SANFORD, K. K., LIKELY, G. D., and EARLE, W. R.: The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells *in vitro*. J. Nat. Cancer Inst. 15: 215–230, 1954.

(4) PARKER, R. C.: Cultivation of tumor cells *in vitro*. Canad. Cancer Conf. 1: 42–54, 1955.

(5) MOORE, A. E., SOUTHAM, C. M., and STERNBERG, S. S.: Neoplastic changes developing in epithelial cell lines derived from normal persons. Science 124: 127–129, 1956.

(6) LEIGHTON, J., KLINE, I., and ORR, H. C.: Transformation of normal human fibroblasts into histologically malignant tissue *in vitro*. Science 123: 502–503, 1956.

(7) BERMAN, L., and STULBERG, C. S.: Eight culture strains (Detroit) of human epithelial-like cells. Proc. Soc. Exper. Biol. & Med. 92: 730–735, 1956.

(8) HSU, T. C., and MOORHEAD, P. S.: Mammalian chromosomes *in vitro*. VII. Heteroploidy in human cell strains. J. Nat. Cancer Inst. 18: 463–471, 1957.

(9) LAMBERT, R. A.: Comparative studies upon cancer cells and normal cells. II. The character of growth *in vitro* with special reference to cell division. J. Exper. Med. 17: 499–510, 1913.

(10) LEWIS, M. R.: Nuclear structures of malignant cells. (Abstract.) Anat. Rec. 45: 268–269, 1930.

(11) ———: Behavior of the chromosomes in the cells of malignant growths. (Abstract.) Anat. Rec. 52: 65, 1932.

(12) LEWIS, M. R., and LOCKWOOD, J.: Tetraploid number of chromosomes in malignant cell of Walker rat sarcoma No. 1. Bull. Johns Hopkins Hosp. 44: 187–198, 1929.

(13) LEWIS, W. H.: Normal and malignant cells. Science 81: 545–553, 1935.

(14) ———: Malignant cells. Harvey Lect. 31: 214–234, 1936.

(15) LEWIS, W. H., and LEWIS, M. R.: Behavior of cells in tissue culture. *In* General Cytology (COWDRY, E. V., ed.). Chicago, Univ. Chicago Press, 1924, pp. 383–448.

(16) YERGANIAN, G.: The striped-back or Chinese hamster, *Cricetulus griseus*. J. Nat. Cancer Inst. 20: 705–727, 1958.

(17) ———: Cytogenetic possibilities with the Chinese hamster, *Cricetulus barabensis griseus*. (Abstract.) Genetics 37: 638–639, 1952.

(18) Tissue Culture Course: An Introduction to Cell and Tissue Culture. Minneapolis, Burgess Publishing Co., 1955, pp. 2–4 and 22–24.

(19) FOLEY, G. E., and HANDLER, A. H.: Differentiation of "normal" and neoplastic cells maintained in tissue culture by implantation into normal hamsters. Proc. Soc. Exper. Biol. & Med. 94: 661–664, 1957.

PLATES

PLATE 45

Phase-contrast photographs of living cultures in T-15 flasks. × 200

FIGURE 1.—Spindle cells of line C.

FIGURE 2.—Flattened polyhedral cells of line P.

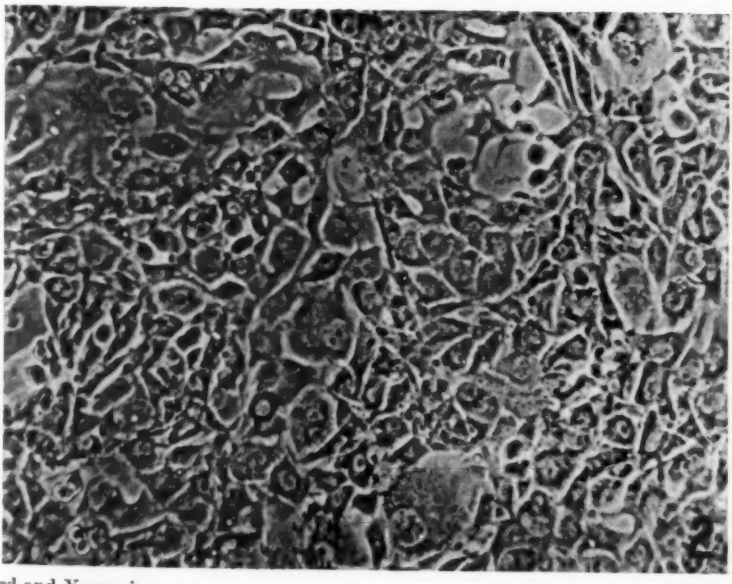


PLATE 46

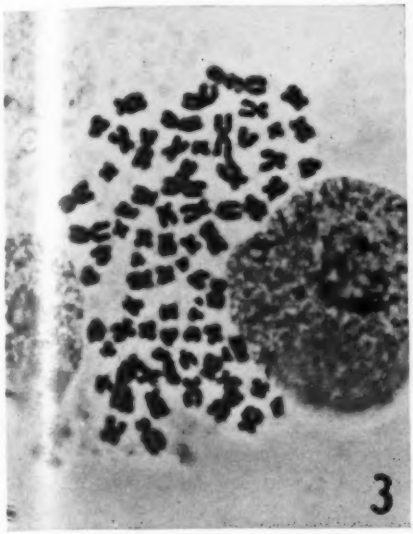
All figures, except figure 6, in plates 46 to 52 are phase-contrast photographs of orcein-stained metaphase plates, or their corresponding diagrams.

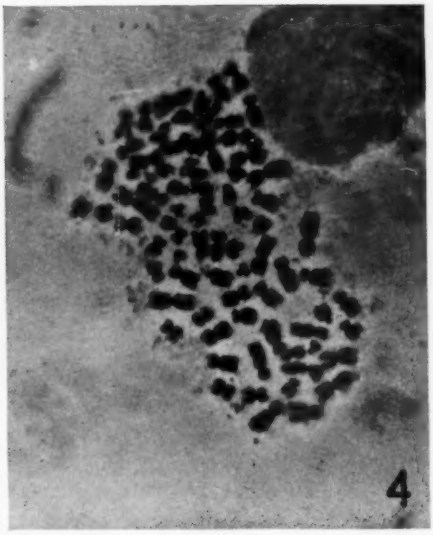
FIGURE 3.—"Changed" hamster A metaphase: 75 chromosomes. × 1500

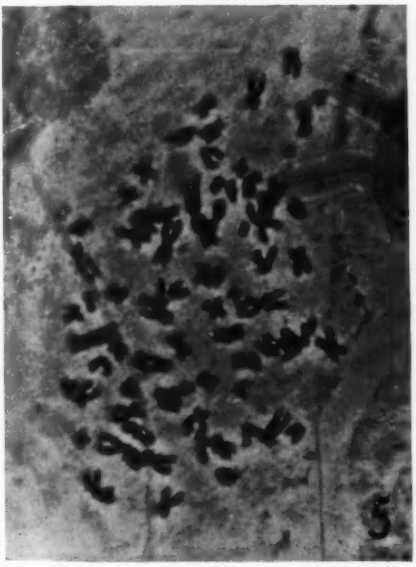
FIGURE 4.—Chang conjunctival cell metaphase: 82 chromosomes. × 1500

FIGURE 5.—HeLa cell metaphase: 74 chromosomes. × 1500

FIGURE 6.—Hematoxylin and eosin-stained section of line A cheek-pouch nodule showing 7 mitoses. × 400


3


4


5

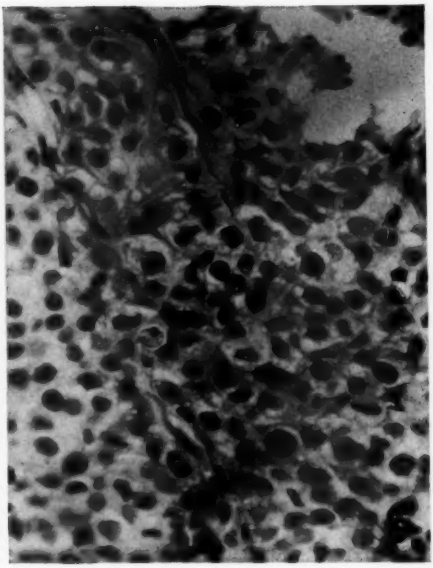

6

PLATE 47

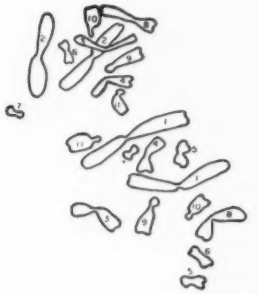
FIGURE 7.—Diploid V metaphase: 22 chromosomes: "Classical" Chinese hamster pattern. × 2500

FIGURE 8.—Diagram of figure 7.

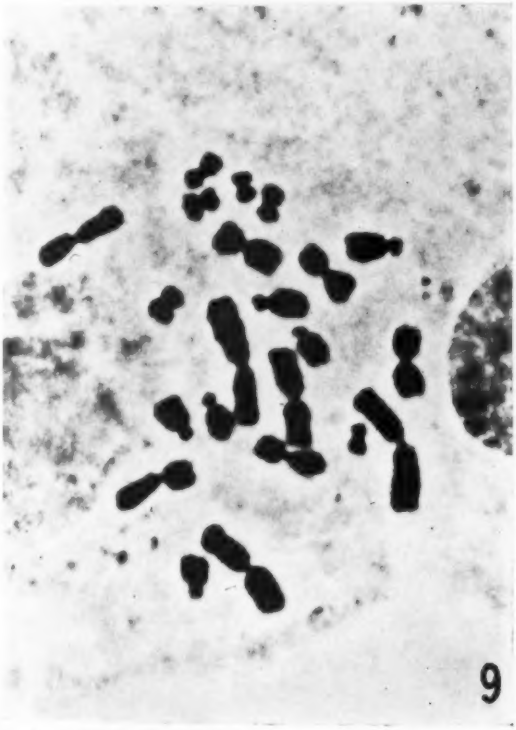
FIGURE 9.—Diploid V metaphase: 22 chromosomes: "Classical" Chinese hamster pattern. × 2500

FIGURE 10.—Diagram of figure 9.

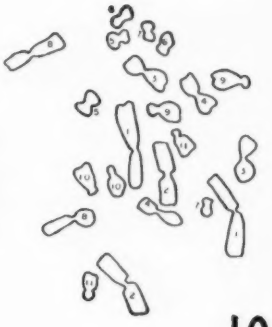
7



8



9



10

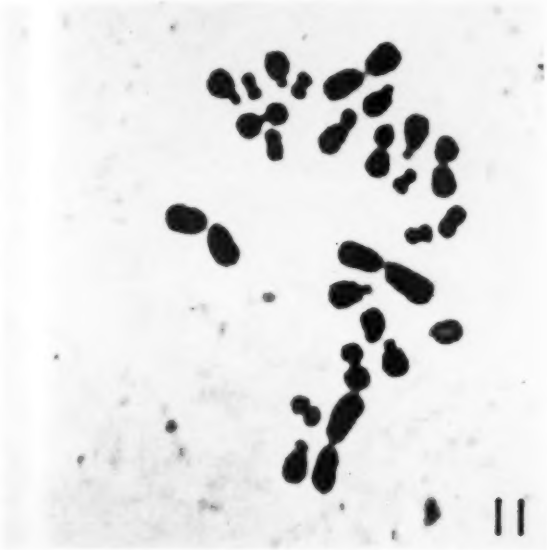
PLATE 48

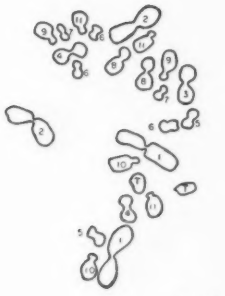
FIGURE 11.—Hyperdiploid C metaphase: 23 + 2 atypical telocentric chromosomes; absent #3, 4, or 8, extra #6 or 7, extra #10 or 11, 2 telocentrics. × 2500

FIGURE 12.—Diagram of figure 11: atypical telocentric chromosomes labeled "T" in this and subsequent diagrams.

FIGURE 13.—Hyperdiploid C metaphase: 23 + 2 atypical telocentric chromosomes; absent #3, 4, or 8, extra #6 or 7, extra #10 or 11, 2 telocentrics. × 2500

FIGURE 14.—Diagram of figure 13.


11


12

13

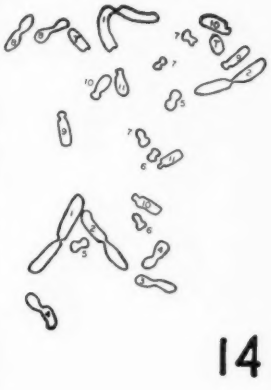

14

PLATE 49

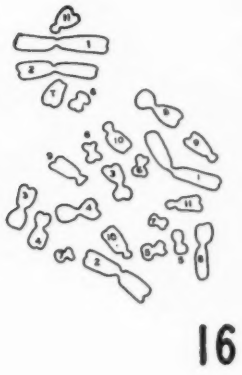
FIGURE 15.—Hyperdiploid C metaphase: 23 chromosomes + 1 atypical telocentric; normal complement with extra #6 or 7, and 1 telocentric. × 2500

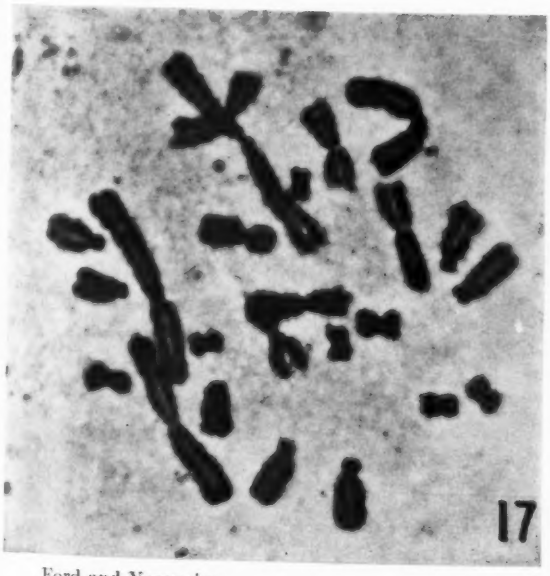
FIGURE 16.—Diagram of figure 15.

FIGURE 17.—Hyperdiploid C metaphase: 23 chromosomes + 1 atypical telocentric; normal complement with extra #6 or 7, and 1 telocentric. × 2500

FIGURE 18.—Diagram of figure 17.

15


16


17

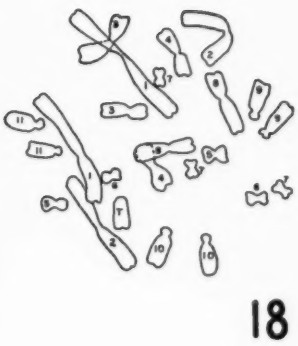

18

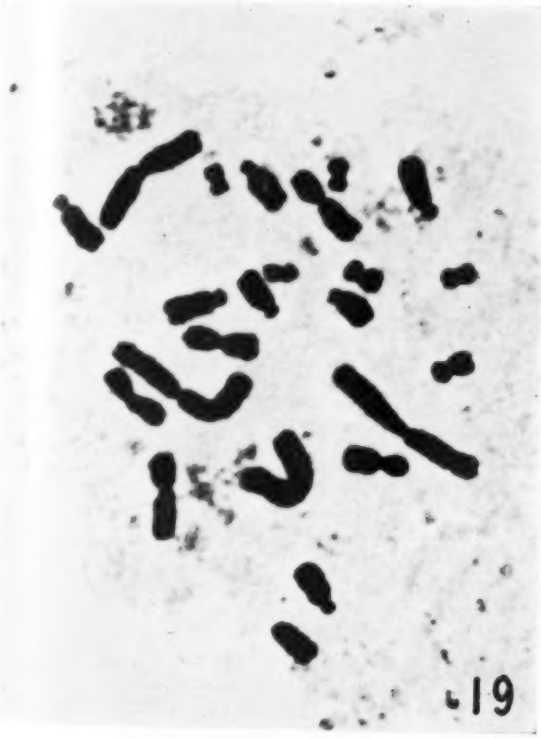
PLATE 50

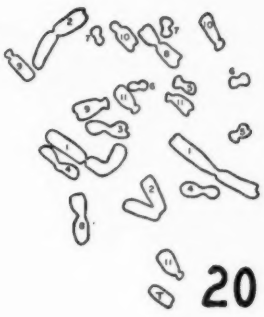
FIGURE 19.—Diploid P metaphase: 22 chromosomes + 1 atypical telocentric; absent #3 or 4, extra #11, 1 telocentric. × 2500

FIGURE 20.—Diagram of figure 19.

FIGURE 21.—Diploid P metaphase: 22 chromosomes + 1 atypical telocentric; absent #3, extra #10 or 11, 1 telocentric. × 2500

FIGURE 22.—Diagram of figure 21.


19


20

21

22

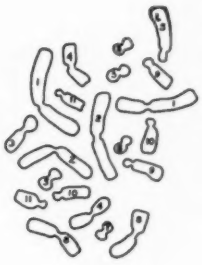
PLATE 51

FIGURE 23.—Hypodiploid metaphase from methylcholanthrene-treated C subline: 21 chromosomes; large subterminal chromosome (LS), short arm to one of the #1 chromosomes, absent #3, 4, or 8, absent #6 or 7. × 2500

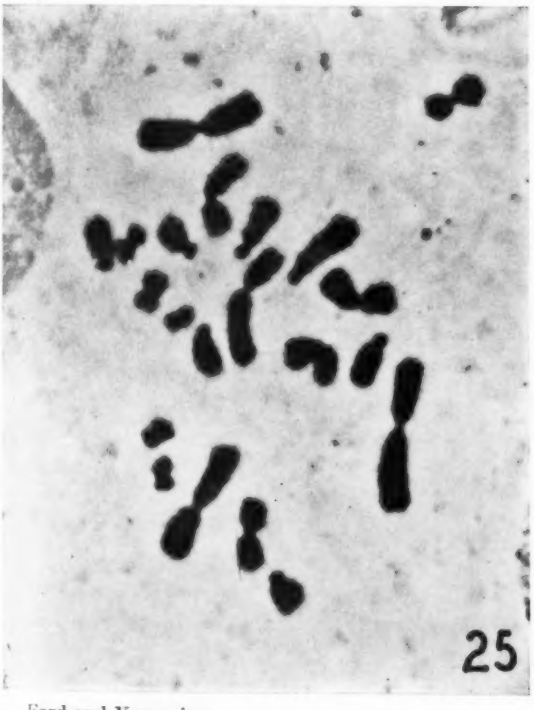
FIGURE 24.—Diagram of figure 23.

FIGURE 25.—Hypodiploid metaphase from methylcholanthrene-treated C subline: 21 chromosomes; large subterminal chromosome (LS), short arm to one of the #1 chromosomes; absent #3, 4, or 8, absent #6 or 7. × 2500

FIGURE 26.—Diagram of figure 25.



24



26

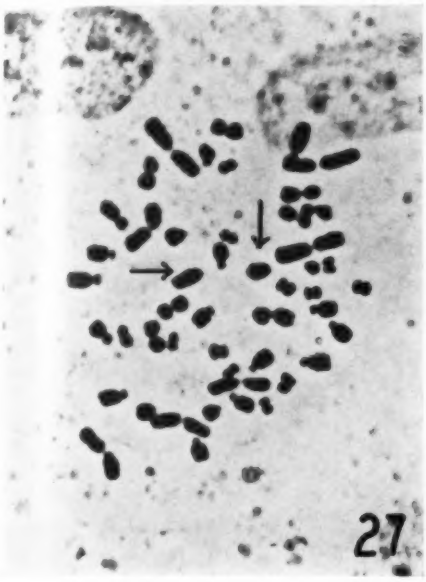
PLATE 52

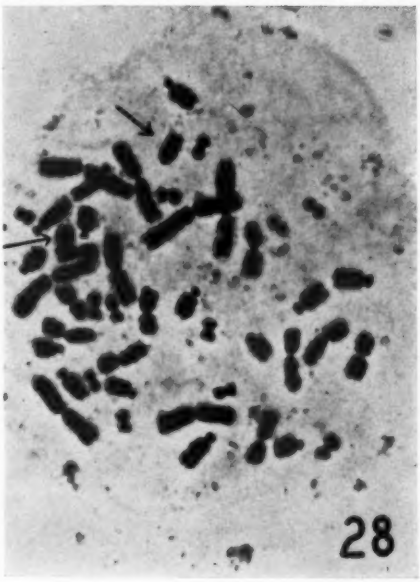
FIGURE 27.—Hypertetraploid C metaphase: 46 + 2 telocentric chromosomes; atypical telocentric chromosomes marked with *arrows*. × 1500

FIGURE 28.—Tetraploid P metaphases: 44 + 2 telocentric chromosomes; atypical telocentric chromosomes marked with *arrows*. × 1500

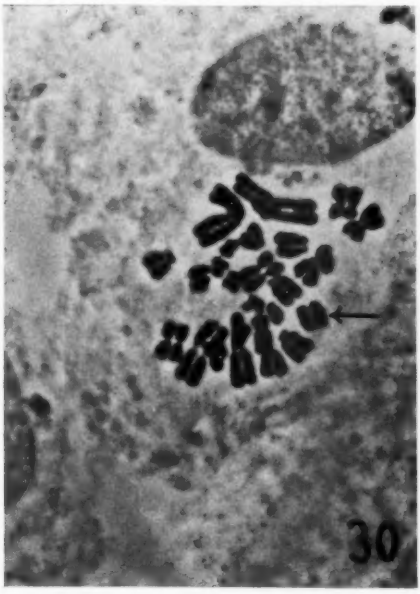
FIGURE 29.—Hypertriploid metaphase from methylcholanthrene-treated C subline: 35 chromosomes; 2 large subterminal chromosomes marked with *arrows*. × 1500

FIGURE 30.—Hyperdiploid C endomitosis: 26 reduplicated chromosomes; reduplication of atypical telocentric chromosome marked with *arrow*. × 1500


27


28

29


30

Announcements

Two-Week Course in Cancer Chemotherapy, October 20–31, 1958

The Sloan-Kettering Institute for Cancer Research, the Memorial and James Ewing Hospitals, and the Sloan-Kettering Graduate Division of Cornell University Medical College will give a course that will include lectures and demonstrations of screening methods, pharmacological techniques, methods for the clinical evaluation of potential chemotherapeutic agents and a review of the chemistry, pharmacological effects, and clinical applications of the polyfunctional alkylating agents, the antimetabolites, steroid hormones, miscellaneous agents in the treatment of cancer, and other selected topics. The course is principally designed for physicians interested in cancer chemotherapy. Applications, including a brief summary of the applicant's clinical training and current appointments should be made to Dr. C. P. Rhoads, Sloan-Kettering Institute, 410 East 68th Street, New York 21, New York.

Enrollment is limited, and application should be made as soon as possible. The fee for this course, which includes a syllabus covering the subject matter presented, is $25.00 payable at the time of registration.

Symposium on Fundamental Cancer Research

"Genetics and Neoplastic Growth" will be the topic for the Thirteenth Annual Symposium on Fundamental Cancer Research, to be held February 26, 27, and 28, 1959, at the University of Texas M. D. Anderson Hospital and Tumor Institute, Houston.

General Chairman, Felix L. Haas has announced that five sessions have been arranged and session chairmen named. They include:

Fundamental Aspects of Genetics in Carcinogenesis I. Wilson S. Stone, The University of Texas, Austin, Chairman

Fundamental Aspects of Genetics in Carcinogenesis II. M. Demerec, Carnegie Institution, Cold Spring Harbor, New York, Chairman

Gene Interaction in Neoplastic Growth. T. S. Painter, The University of Texas, Austin, Chairman

Genetic Basis of Cell Resistance. Walter E. Heston, National Cancer Institute, U. S. P. H. S., Bethesda, Chairman

Heredity and Human Cancer. James F. Crow, University of Wisconsin, Madison, Chairman

For information regarding the program, inquiries should be addressed to the General Chairman: Felix L. Haas, Department of Biology, M. D. Anderson Hospital, Houston 25, Texas.